# SOME ASPECTS OF RESISTANCE TO ARSENICAL DRUGS IN TRYPANOSOMA BRUCEI BRUCEI

by

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This thesis is specially
dedicated to my wife and
also to my parents.

CONTENTS

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Abstract of the thesis

1. A strain resistant to melarsen oxide was produced from a laboratory adapted line of Trypanosoma brucei using the short passage method. It was necessary to passage the original line of trypanosomes 18 times with 16 exposures to the drug to reach a resistance factor of 13. With the help of a drug sensitivity test, using the oxygen electrode and Warburg manometry, it was found that the new strain is a heterogeneous population of trypanosomes with respect to the sensitivity to melarsen oxide. The resistant character is stable after serial passage in rats.

2. Different alternatives were suggested and investigated in order to rationalise the development of resistance in biochemical terms. The alternatives were:

- Modification of metabolism to bypass the action of the drug.
- Modification of enzyme targets with regard to different kinetic parameters, increased specific activities and different sensitivities to the drug.
- Modification of the drug transport.

3. The metabolic studies showed that although the glycolytic sequence is the basis of metabolic route in both parasites, the resistant strain shows lower pyruvate/glucose and pyruvate/oxygen ratios from glucose catabolism when compared with the parent line. Furthermore some $CO_2$ is produced by the resistant strain with an RQ value of 0.1 (approx.). These characteristics are observed in the absence of the drug. The patterns of inhibition of the oxygen consumption with whole cells from the parent line and the resistant strain were different. The $I_{50}$ value for the parent line was $6.25 \times 10^{-6}$ M melarsen oxide and only 25 to 30% inhibition is observed for the same parameter at the highest concentration of drug used ($250 \times 10^{-6}$ M) in the resistant organism. The patterns of inhibition in water lysates however are apparently the same in the parent line and the resistant strain; this feature is

interpreted in terms of a permeability barrier for the drug in the drug fast trypanosomes. The general carbon balance with whole cells from the parent line was severely affected under the influence of the drug as a sharp reduction was observed in the pyruvate/glucose and pyruvate/oxygen ratios from glucose catabolism. This corroborates data already reported, suggesting that pyruvate kinase (E.C. 2.7.1.40) is the focal point of action of organic arsenicals. When the same parameters were investigated in whole cells from the resistant strain a minor inhibition of the metabolism was found, with the difference that the metabolic ratios remained constant in the presence of the drug; furthermore the $CO_2$ production described in the absence of the drug was abolished. Therefore it is suggested that although some common site of inhibition is still affected in the drug fast organisms, there is also the possibility of a minor pathway for the further metabolism of pyruvate when the drug is present.

4. The enzymological studies suggest that pyruvate kinase already reported as the main target of arsenicals in trypanosomes has the same kinetic characteristics in the parent line and the resistant strain. The $S_{50}$ values with respect to phosphoenolpyruvate were $1.60 \pm 0.30 \times 10^{-3}$M; Hill coefficient = $2.16 \pm 0.30$. The $K_m$ value with respect to ADP was $3.17 \pm 0.22 \times 10^{-3}$M. The $V_{max}$ values in terms of mg of protein were also the same in the parent and resistant line. Phosphoglycerate kinase (E.C. 2.7.2.3) another target of organic arsenicals did not show any modification as a result of the development of resistance. The enzyme showed apparent hyperbolic responses with respect to ATP and phosphoglycerate; upon linear transformation of the data, ATP showed biphasic linear responses with $K_{m_1}$ value = $0.89 \pm 0.20 \times 10^{-3}$M and $K_{m_2}$ value = $0.30 \pm 0.9$. Phosphoglycerate showed a simple linear response with $K_m$ value = $5.80 \pm 0.42\ 10^{-3}$M. The $V_{max}$ values in terms of

mg of protein were also the same. Pyruvate kinase and phosphoglycerate kinase in resistant strain showed similar patterns of inhibition with respect to melarsen oxide when compared with the parent line ($I_{50}$ value for pyruvate kinase = 7.60 $10^{-5}$M melarsen oxide; $I_{50}$ value for phosphoglycerate kinase = 0.53 x $10^{-3}$M melarsen oxide.)

5. When drug transport studies were performed it was found that melarsen oxide gets into the parasite via a carrier mediated mechanism with the following kinetic parameters, calculated using a non-linear correlation: $K_t$ value =14.42.$10^{-6}$M; $V_{max}$=5.19 nmoles $min^{-1}$ mg $protein^{-1}$; Hill coefficient = 2.27. It was found that the resistant strain lost the carrier mediated transport mechanism and only a diffusion component, assumed to be present in the parent line, remains responsible for a low rate of uptake. The transport of melarsen oxide in the parent line is concentrative, inhibited by the analogue melamine ($K_i$ 7.0.$10^{-6}$M), temperature dependant, inhibited by iodoacetate and possibly involves -SH groups as indicated by the lack of uptake of the pentavalent sodium melarsen.

Uptake studies using different arsenicals suggest that there is a single site of uptake for the drugs studied and that resistance in the new strain is the result of a modification of that part involved in the binding of the melaminyl residue of melarsen oxide at the uptake site.

ABBREVIATIONS

| | |
|---|---|
| AgDDC | silver diethyl dithiocarbamate |
| BEU | Biochemistry Edinburgh University |
| BSA | bovine serum albumin |
| DHAP | Dihydroxyacetone phosphate |
| DTNB | 5',5' dithiobis(2 nitrobenzoic acid) |
| DTT | Dithiothreitol |
| EDTA | Ethylenediaminetreta-acetic acid |
| FAO | Food and Agricultural Organization |
| F6P | Fructose-6-phosphate |
| FDP | Fructose-1,6-diphosphate |
| GP | glycerol-3-phosphate |
| G3P | glycerate-3-phosphate |
| GK | Glycerol kinase |
| IAA | Iodoacetate |
| ISS | Intermediate short stumpy |
| LDH | lactate dehydrogenase |
| LS | Long slender |
| PEP | Phosphoenolpyruvate |
| $P_i$ | Phosphate |
| PGK | Phosphoglycerate kinase |
| PK | pyruvate kinase |
| RQ | Respiratory quotient |
| SS | Short stumpy |
| TEA | triethanolamine |
| TREU | Trypanosoon: Research Edinburgh University |
| WHO | World Health Organisation |

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# 1. INTRODUCTION

## 1.1 General introduction

### 1.1.1 Natural history of sleeping sickness and drug resistance

Human trypanosomiasis (sleeping sickness) is recognised today as a major health, economic and social problem in Africa. The trypanosomes of man and the counterparts in domestic animals, with the consequent problems caused by them, are a major barrier to economic and agricultural development in large areas of the tropical sub-continent. In practice it has been found that chemotherapy plays a major role in the control of human and animal trypanosomiasis. The causative organisms and the disease, which have been studied since the beginning of the century, are of sufficient importance that today they are considered main features of the Special Programme for Research and Training in Tropical Diseases, sponsored by the World Health Organisation (WHO, 1976).

Trypanosomes of the brucei group are the causative organisms of sleeping sickness. Members of this group are of wide distribution, especially in Africa, and throughout the world (Ormerod, 1979). Trypanosoma brucei rhodesiense (hereafter referred to as T. rhodesiense) and T. brucei gambiense (T. gambiense) are responsible for the acute and chronic forms of the human disease respectively. Probably both subspecies of trypanosome which infect man in Africa evolved from Trypanosome brucei brucei (T. brucei) or at least from a common ancestral species (Baker, 1963). Normally, an infectious disease such as sleeping sickness, changes its natural history as parasite and host establish a genetic balance based on the invasiveness of the parasite and the resistance of the host, when an insect transmitting agents are also involved (Ormerod, 1970).

Different approaches to restrain the disease have been taken either by controlling the insect vector or the parasite. In the first category both tse-tse eradication with insecticides (Davis, 1971) and insect population control with sterile males (Dame, 1970) have been tried.

The former is the most common and practical method in the field (Burnett, 1970). Vector eradication by insecticides, if 100% successful, could interrupt the transmission cycle. The vulnerable aspect of vector control by insecticides, however, is the risk of reinfestation. Biological control by the release of sterile male tse-tse flies is being investigated, the principal constraints being the development of suitable systems to produce colonies of flies and the reduction of the population of insects to manageable densities prior to the release of sterile males (WHO, 1976). For many years it has been accepted that the best way to control sleeping sickness is to form permanent agricultural settlements so that the tse-tse fly is excluded from inhabited areas and is not constantly around the houses (Wilcocks, 1962).

Another important approach to the control of sleeping sickness is that of selective chemotherapy and removal of the trypanosomes from man or infected animals. Chemotherapeutic control of African trypanosomes relies on only a few active structures, four drug classes now being available: suramin, melarsoprol and nitrofurazone for curative purposes and pentamidine for prophylaxis. All these drugs have serious side effects.

Drug resistance to most chemotherapeutic agents is a widespread phenomenon. It has been suggested that it is as old as chemotherapy itself (Schnitzer and Grunberg, 1957). In 1907, when Ehrlich reported the trypanocidal action of rosaniline he also included Franke and Roehl's observation that the trypanosomes could be rendered resistant to the dyestuff. Resistance to melaminyl arsenicals, of which melarsoprol is a representative, is a matter of serious concern. It has been reported recently that resistance to melarsoprol is occurring with increasing frequency (WHO/FAO, 1979). Apart from the work of Damper and Patton (1976a,b) on the mechanism of resistance to pentamidine,

little or inconclusive evidence is available regarding resistance to other drugs like organic arsenicals and specifically the melaminyl arsenicals. It is for this reason that the main objective of this thesis was to gain some insight into the mechanism of development of resistance to these active substances in T. brucei.

### 1.1.2 Taxonomical position of T. brucei

The systematic position of trypanosomes among protozoa, with special reference to the African organisms, has been discussed rigorously by Hoare, (1970). The genus Trypanosoma has been divided into two sections: salivaria and stercoraria, in which the different subgenus are arranged (Hoare, 1964). This subdivision is based on the development of the parasite in the mammalian host, the subgenus Trypanozoom to which the Brucei group belongs, being classified in the first section. Trypanosomes of the Brucei group may be regarded as a complex species, in which there are different groups of organisms with different modes of transmission, different degrees of virulence and epidemiology; these characteristics however, are not sufficient to justify a classification higher than the subspecific level (Ormerod, 1967).

The proposals for the nomenclature of salivarian trypanosomes and for the maintenance of a reference collection, as suggested by the WHO (1978), were used throughout this work. An attempt has been made to present the pedigree of newly developed resistant lines, using this nomenclature.

### 1.1.3 Life cycle and morphology

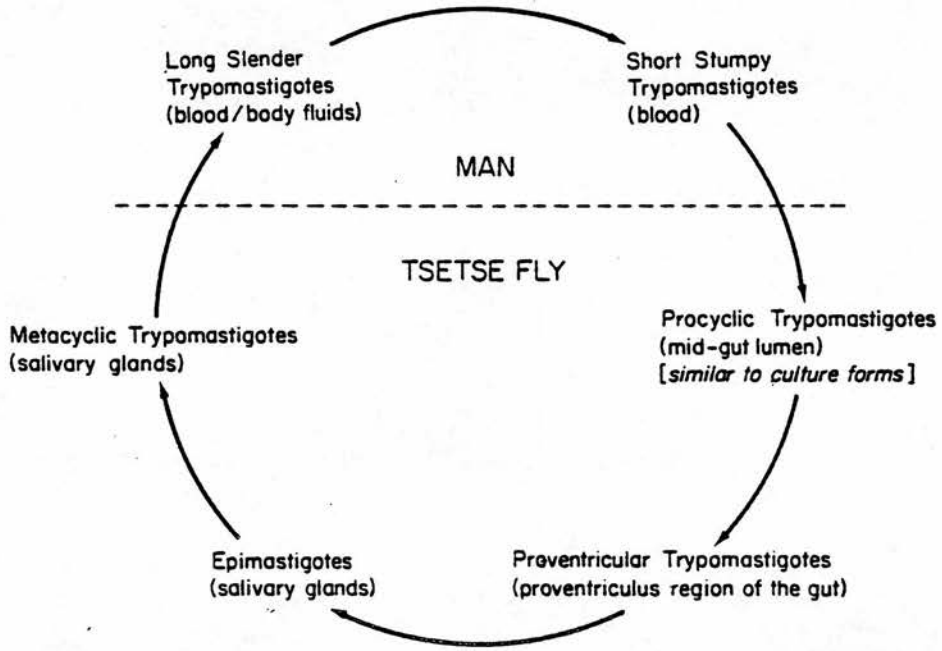
A complete account of the development of resistance in these parasites should consider the possibility of modification of some aspects of their physiology throughout the life cycle. The possibility is greater for the stages of the life cycle in the mammalian host,

Table 1.1: Comparison of the metabolic status of the different forms of *T. brucei*.

| Parameter | Blood trypomastigote forms | | Culture procyclic Trypomastigote forms |
|---|---|---|---|
| | Long slender | Short stumpy | |
| glycolysis | + | + | + |
| TCA cycle | - | Partial | + |
| L-α-glycerophosphate oxidase | + | + | Low |
| Proline oxidase | - | Low | + |
| Cytochrome oxidase | - | - | + |
| Cyanide sensitivity | - | - | Partial |
| Mitochondria | Very few tubular cristae | Tubular cristae | Plate like cristae |
| Growth temperature ($^{o}$C) | 37 | 37 | 25 |

+, present -, absent. Based on Table 1 of Gutteridge and Rogerson (1979).

Figure 1.1: The life cycle of T. brucei.




Taken from Gutteridge and Coombs (1977).

when the trypanosomes are in direct contact with the drug. No information is available on the possible participation of the insect stages and the role of the insect vector on the development of resistance. In any case transmission by the insect vector does not seem to impair the stability of drug resistance in trypanosomes (Murgatroyd and Yorke, 1937, Gray and Roberts, 1968).

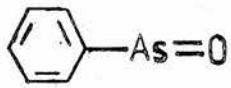
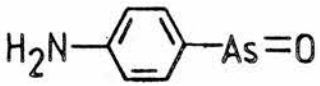
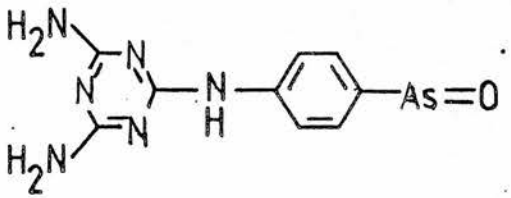
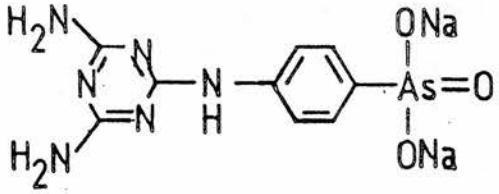
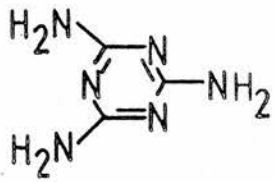
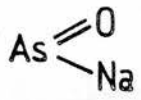
Infections with T. brucei, show a marked variability, in size and shape of the trypomastigotes circulating in the blood (pleomorphism). The three main different forms are the long slender (L.S.), the short stumpy (S.S.) and the intermediate (I.S.S.) form (Hoare, 1970, 1972). The development of different forms is not confined to the stages in the mammalian host but also occurs in the insect vector, where the S.S. trypomastigotes or preadapted forms for the invertebrate undergo further morphological and biochemical change (see Figure 1.1). An account of the biochemical significance of these morphological changes is seen in Table 1.1, adapted from Gutteridge and Rogerson (1979). Finally a monomorphic, laboratory adapted line of T. brucei was used in the experimental section of this thesis. It should be noted that the arsenicals are equally effective against monomorphic and pleomorphic lines (Flynn and Bowman, 1973).

### 1.1.4 Development of the life cycle in vitro

One of the greatest obstacles to studying the physiological changes associated with drug resistance in trypanosomes is the inability to cultivate these organisms in vitro (Bishop, 1959). The present status of the in vitro cultivation of animal infective African trypanosomes (where the phenomenom of drug resistance occurs regularly) was reviewed recently by Hirumi et al., (1979). The continuous propagation of haematozoic trypomastigotes of T. brucei and T. rhodeniense, retaining their infectivity for the mammalian host is now possible (Hirumi et al.,

1977a,b, Hill et al., 1978). Although, there are some chemical and physical parameters that remain to be fully characterised, in vitro cultivation of these parasites is a potentially important tool for the study of the development of drug resistance under fully standardised conditions. However the original statement of Bishop, still obtains in spite of recent developments in this area of the biology of the parasites.

Table 1.2: The structure of some organic arsenicals and other related structures.

| Structure | Formal name | Common name |
|---|---|---|
|  | phenylarsonous acid | phenyl arsenoxide |
|  | p-Aminophenyl arsenoxide | reduced atoxyl |
|  | p-(2,4-diamino-s-triazinyl-6)-aminophenyl arsenoxide | melarsen oxide |
|  | disodium p-(2,4,6-diamino-s-triazinyl-6)-aminophenyl arsonate | sodium melarsen |
|  | 2,4,6-triamino-s-triasine | melamine |
|  | sodium arsenite | sodium arsenite |

## 1.2 The action of organic arsenicals and drug resistant in trypanocides

### 1.2.1 Terminology of arsenical drugs

The nomenclature of Doak and Freedman (1960) has been adopted in this work. The names and structures used are shown in Table 1.2. The structures do not necessarily represent the form in solution. Some information is available on the dominant forms of organic arsenicals in solution but by no means is there complete agreement on pka values and the extent of hydration. A complete account will be found in Webb (1966).

### 1.2.2 Development of the melaminyl arsenicals

The development of the chemotherapy and chemoprophylaxis of trypanosomiasis in man has encountered many technical problems; the particular case of aliphatic phenyl arsonic acids and their reduced counterparts can be used to exemplify this point, the development of resistance being a major drawback to their utilisation. Differences in sensitivity between different lines of T. rhodesiense and T. gambiense also made these drugs ineffective in some cases. Finally the toxicity of these structures made them unsuitable for widespread use. A need for new compounds active against African trypanosomes was evident by the middle of this century and a unique class of phenyl arsonates and arsenoxides, in which melamine (2,4,6-triamino-s-triazine) is coupled to the para-position of the metal containing substituent was developed by Friedheim, (1940). The new pentavalent arsenical,named "melarsen" was found to be very effective against late-stage human trypanosomiasis. The trivalent analogue melarsen oxide (Friedheim, 1944) was very potent against the flagellates in vivo and in vitro (Rollo and Williamson, 1949). Unfortunately, according to the latter authors, the toxicity of melarsen oxide precludes its general use.

The activity of melarsen against tryparsamide resistant T. gambiense is of considerable practical importance as it was found that the

compound was not only active in the laboratory (Williamson and Lourie, 1948) but also in the field (Van Hoof, 1947). Also medication with tryparsamide and other pentavalent arsenicals may be responsible for the development of ocular lesions under experimental conditions (Hurst, 1959). Melaminyl arsenicals are however apparently unable to produce optic lesions. The organic substituents therefore appear to have some specific influence on the uptake of these drugs by the central nervous system (CNS) (Williamson, 1962; 1970).

The development of B.A.L. (dimercaprol or 2,3 dimercaptopropanol) by Peters et al., (1945) as a detoxifying agent for arsenicals by the formation of a stable ring compound with the trivalent arsenic, led to the development of different organic arsenoxides. The first was the less toxic compound melarsoprol (Mel B) and the second an even less toxic and more water soluble derivative, melarsonyl potassium (Mel W) (Friedman and De Jongh, 1959). The main advantage in the use of melaminyl arsenicals was their ability to cross the blood-brain barrier and to show activity against the late stages of infection with CNS involvement (Williamson, 1962). This characteristic seems also to depend on the drugs ionisation state and lipid solubility (Friedheim and Vogel, 1947).

In spite of the problems associated with drug resistance and toxicity, Mel B is widely established as the drug of choice in the treatment of the later stages of sleeping sickness. Nitrofurazone is employed in melarsoprol resistant cases (WHO/FAO, 1979).

It has been suggested (Flynn, 1971) that these disulphide derivatives Mel B and Mel W are hydrolysed to the arsenoxide form, before they can be active therapeutically. Because of the evolution of these compounds from melarsen oxide and the necessary reversion to the original structure for activity, melarsen oxide has been used in the experimental part of this work, as a model for the melaminyl arsenicals.

### 1.2.3 The action of arsenicals on metabolism

Organic compounds of arsenic have been used as toxic agents for several hundred years. The toxicity of these compounds made them particularly popular as suicidal and homicidal poisons of widespread use during the 16th and 17th century. As a result of their potent action much effort has been directed towards elucidating the mechanisms of action of these compounds.

It has been known for a relatively long time that inorganic arsenite has a lethal effect on trypanosomes but its toxicity precluded general use. Thomas and Breinl, (1905) realised the value of sodium arsanilate in the control of murine trypanosomiasis and at the same time, the curative effect of this compound on human sleeping sickness was reported. From the beginning there has been disagreement about the action of arsenicals *in vivo* and *in vitro*. For example, the above observation did not correlate with that of Ehrlich (1909) who reported that sodium arsanilate was inactive *in vitro*. Thanks to the variety of results obtained and to the antagonistic conclusions put forward a complete generation of organic arsenicals was developed for the treatment, not only of trypanosomiasis, but also of syphilis and of amoebiasis. These new compounds are now well known as enzymatic and metabolic poisons in mammalian tissues (Webb, 1966).

Ehrlich, (1909) suggested that pentavalent arsenicals must be reduced to the trivalent form *in vivo* to be active and he did not consider the mechanism of action to involve the release of inorganic arsenite. A very important phenomenon reported since the appearance of organic arsenicals was that certain microorganisms become increasingly resistant to these drugs during continuous exposure (see Webb, 1966).

Some of the most important concepts in chemotherapy were suggested as a result of the work with organic arsenicals. Ehrlich recognised the

importance of the quantitative measurement of the relationship between the dose of a compound and the therapeutic effect produced by it. Another concept developed was the existence of an arseno-receptor in the cell, required for the drug, to exert any biological action. It was later found that these specific receptors within the cell were thiol (-SH) groups (Voegtlin et al., 1923 and Barber, 1929). The original concept of arsenicals as "protoplasmatic poisons", suggesting that arsenicals exert their activity by non-specific coagulation of proteins (Ehrlich, 1909) was therefore untenable. The idea of metabolic inhibition was gaining ground and a clearer picture emerged, although without definitive conclusions.

Krebs, (1933a,b) showed that arsenite caused a marked accumulation of pyruvate and other ketoacids during the metabolism of amino acids by kidney preparations. It was evident that the oxidation of ketoacids was specifically blocked by arsenicals and, this observation was the first evidence of the primary metabolic target in the mammalian model. More evidence with different tissues has substantiated this hypothesis. The work of Peters et al., (1946) and Peters, (1955) demonstrated without question that pyruvate oxidase and the oxidation of pyruvate are extremely susceptible to trivalent arsenicals and this also suggested the participation of thiol groups in the mechanism of the reaction.

However the mechanism of action of organic arsenicals on the metabolism of trypanosomes remained a mystery for many years as it was shown that monomorphic bloodstream trypanosomes lack the α-ketoacid oxidase systems (Ryley, 1956, Grant and Fulton, 1957, Flynn and Bowman, 1973). Hence there must be an alternative site of action for arsenicals. It is now known that there is a definitive preference for pyruvate kinase, which has been pin-pointed as a major focal point for the

trypanocidal activity of organic arsenicals, melarsen oxide being one of the most important (Flynn, 1971). It has also been shown in this thesis that the enzyme phosphoglycerate kinase of T. brucei is inhibited by different arsenicals. The main site of action pyruvate kinase is constant for the pleomorphic and monomorphic forms of the parasite (Flynn and Bowman, 1974). A more detailed account on the action of organic arsenicals, including the melaminyl arsenicals, is given in subsection 2.1.6.

With regard to the therapeutic effect of these compounds, the earliest arsenicals used were usually pentavalent arsonic acids. The same compounds were found to be inactive in vitro so reduction is considered necessary, as the arsenoxides are the recognised enzyme and metabolic inhibitors (Webb, 1966). It has also been suggested that the organic part of the molecule does not participate directly in the reaction with the target but serves to modify the properties of the drug molecule, especially with regard to the penetration of cell membranes. More evidence on the state of oxidation of arsenicals showed that atoxyl and other related arsonic acids are not readily reduced to arsenoxides in the body. However, local reduction by the parasite remains a possibility (Crawford and Levy, 1947; Frost, 1967). This situation remains unclear as Lourie et al., (1935) reported reduction of tryparsamide to a highly active compound when incubated with erythrocytes. To avoid any complications as a result of the state of oxidation of the drug, the trivalent arsenical melarsen oxide is used in this work, this compound being active both in vivo and in vitro.

### 1.2.4 Carbohydrate metabolism in members of the Trypanozoon subgenus

It may be seen that the action of organic arsenicals is closely related to the metabolism of carbohydrates in trypanosomes, it has also

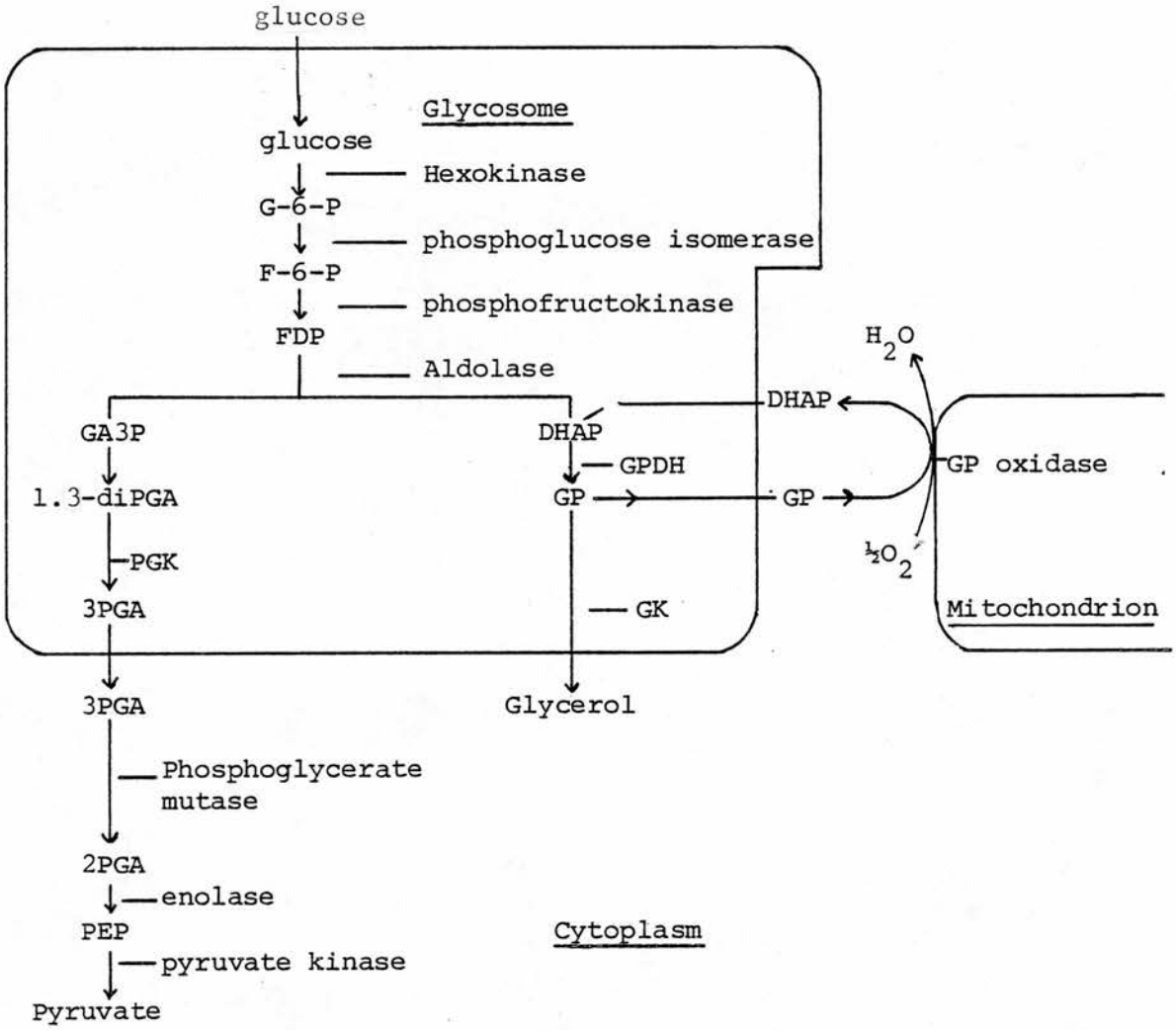
been suggested that a modification of the metabolic pathways involved may be acquired in the development of resistance to these drugs. A brief review is therefore appended of the general metabolic pathway of carbohydrates in members of the Trypanozoon subgenus, because of the involvement of the mechanism of action of arsenicals.

Respiration in bloodstream trypanosomes occurs via a cyanide and CO insensitive pathway, found in all members of the Trypanozoon subgenus (von Brand, 1951; Fulton and Spooner,1959). The members of this group have an absolute dependence on an exogenous supply of carbohydrate. Glucose, fructose, mannose and glycerol will support mobility and respiration of the L.S.form(Ryley, 1962; Bowman and Flynn, 1976). These organisms have no endogenous stores of carbohydrate or any other endogenous source of energy (Ryley, 1956).

The metabolism of carbohydrates is incomplete. Aerobic glycolysis occurs via the Embden-Meyerhof pathway with the difference that it proceeds at a faster rate than in the host tissues. Also, pyruvate is released into the host bloodstream (Ryley, 1956; Grant and Fulton, 1957). Pyruvate cannot be further metabolised as bloodstream forms of L.S. T. brucei do not have α-oxoacid oxidases or a functional citric acid cycle (Flynn and Bowman, 1973). It has been found recently, that the rate limiting step of glycolysis in these parasites is the transport of glucose into the organism (Gruenber et al., 1978).

The NADH generated during glycolysis by the action of glyceraldehyde-3-phosphate dehydrogenase is reoxidised by means of two coupled enzymes; $NAD^+$-dependent L-glycerol-3-phosphate (GP) dehydrogenase and a particulate GP oxidase originally reported by Grant et al., (1961). Catalytic amounts of dihydroxyacetone phosphate (DHAP) are then reduced by NADH to form glycerophosphate, which is the substrate of the GP oxidase. GP is reoxidised to DHAP with the reduction of molecular oxygen.

Figure 1.2: The general metabolism and compartmentation in T. brucei.




The scheme was constructed from results published by Oduro (1977); Opperdoes et al. (1977) and Opperdoes and Borst (1977)

The overall reaction catalysed by these enzyme systems is the transfer of electrons from NADH to molecular oxygen forming water, with a net glycolytic yield of 2 moles of ATP per mole of glucose. Pyruvate is the only end product under aerobic conditions (see Figure 1.2). As reported above pyruvate is not further metabolised because of the absence of a functional Krebs cycle and also the lack of lactate dehydrogenase (Dixon, 1966). Two enzymes are present for the fixation of $CO_2$ in L.S. forms of T. brucei (Klein et al., 1975); malic enzyme and ADP-dependent, PEP carboxykinase have been characterised, pyruvate carboxylase being absent. The metabolic significance of these enzymes is still open to discussion.

Under anaerobic conditions, Grant and Fulton (1957) and recently Hammond (1979), have shown that one mole each of pyruvate and glycerol accumulate per mole of glucose used. DHAP is postulated as the final electron acceptor (Bowman and Flynn, 1976). Originally it was thought that glycerol could be formed by the action of phosphatases on GP as reported by Harvey, (1949) and Gerzeli, (1955). However, there is much evidence to indicate that ATP is synthesised from GP anaerobically (Fulton and Spooner, 1959; Opperdoes et al., 1976; Clarkson and Brohn, 1976; Fairlamb et al., 1977). Recently it has been found that glycerolkinase uses GP and ADP to form ATP and glycerol with a net synthesis of ATP (Hammond, 1979).

The line of T. brucei used in this work corresponds to the characteristics described above.

### 1.2.5 Distribution of glycolytic enzymes and compartmentation in T. brucei

It has been recently shown that in T. brucei many of the glycolytic enzymes are located inside a microbody-like organelle (Oduro, 1977; Opperdoes and Borst, 1977). This localisation is different to the situation in other eukaryotes. The localisation of these enzymes in a

particulate form indicates that the organelle can catalyse the following reactions:

$$\text{glucose} + 2\ \text{DHAP} + 2\ P_i \longrightarrow 2\ (\text{GP} + 3\text{PGA})$$

$$\text{glycerol} + 2\ \text{DHAP} + P_i \longrightarrow 2\ (\text{GP}) + 3\text{PGA}$$

It can be seen that the reactions performed by the glycosome are self sufficient for the production and use of ATP, the reduction of $NAD^+$ and the reoxidation of NADH (see Figure 1.2). PGA leaves the particle and once in the cytoplasm is further metabolised to pyruvate with a net synthesis of ATP. At the same time GP leaves the particle, under aerobic conditions, to be oxidised in the mitochondrion by GP oxidase, these reactions may be summarised as follows:

$$3\text{PGA} + \text{ADP} \longrightarrow \text{Pyruvate} + \text{ATP} + H_2O$$

$$\text{GP} + \tfrac{1}{2}O_2 \longrightarrow \text{DHAP} + H_2O$$

The intracellular localisation of the GP oxidase complex was until recently thought to be in a microbody-like structure (Müller, 1975). However, new evidence supports the idea of a mitochondrial location for the enzyme (Opperdoes et al., 1977). It has also been found that the localisation of glycolytic enzymes in Crithidia fasciculata and in different stages of the life cycle of T. cruzi, is similar to that of T. brucei. It has in fact been suggested that the glycosome is a general feature of the members of the Kinetoplastida (Taylor et al., 1980).

Trypanosomes are, as reported earlier, dependent on glycolysis for ATP synthesis. It has also been suggested that the glycosome has evolved to optimise conditions for glycolysis by creating a compartment where optimal concentrations of enzymes, substrates and cofactors are maintained (Opperdoes and Borst, 1977; Oduro, 1977). Also, it has been reported (Visser and Opperdoes, 1980), that the enzymes within this particle exhibit latency, suggesting the presence of a limiting membrane.

Table 1.3: The action of arsenicals on enzymes in members of the genus Trypanozoon.

| Enzyme | Source | Arsenical | $I_{50}$ concentration mM | Reference |
|---|---|---|---|---|
| Adenosine triphosphtase | T. equiperdum | oxophenarsine | 0.84 | Chen (1948) |
| Glycerol kinase | T. brucei | melarsen oxide | 0.004 | Hammond (1979) |
| L-α-glycerol phosphate oxidase | T. brucei | oxophenarsine | 0.1 (a) | Grant and Sargent (1960) |
| " | " | melarsen oxide | 0.06 | Fairlamb (1977) |
| Hexokinase | T. equiperdum | oxophenarsine | 6.5 (*) | Chen (1948) |
| " | " | tryparsamide | 65.6 (a) | " |
| α-ketoglutarate decarboxylase | T. rhodesiense (SS) | melarsen oxide | 0.02 | Flynn and Bowman (1974) |
| " | " | phenyl arsenoxide | 0.003 | " |
| Phosphofructokinase | T. rhodesiense | oxophenarsine | 0.7 | Jaffe et al. (1971) |
| Phosphoglyceraldehyde dehydrogenase | T. equiperdum | Tryparsamide | 12.0 (*) | Chen (1948) |
| " | " | oxophenarsine | 9.4 (*) | " |
| Phosphoglycerate kinase | T. brucei | melarsen oxide | 0.5 | This work |
| " | " | phenyl arsenoxide | 1.5 | " |
| " | " | reduced atoxyl | 3.6 | " |
| Pyruvate decarboxylase | T. rhodesiense (SS) | melarsen oxide | 0.02 | Flynn and Bowman (1974) |
| " | " | phenyl arsenoxide | 0.004 | " |
| Pyruvate kinase | T. brucei | melarsen oxide | 0.04 | Flynn (1971) |
| " | " | phenyl arsenoxide | 0.19 | " |
| " | " | reduced atoxyl | 0.79 | " |
| Triose phosphate dehydrogenase | T. hippicum | oxophenarsine | 0.01 (b) | Harvey (1949) |
| Phosphoprotein,phospholipids metabolism | T. equiperdum | oxophenarsine | Inhibition present | Cantrell (1954) |

a, $I_{59}$; b, $I_{53}$; *, calculate from the original data.

However it was found that the pool of glycolytic intermediates inside the glycosome equilibrates with the cytoplasmic pool. The possible involvement of the glycosome in the development of resistance was partially evaluated in this work.

1.2.6 Mechanism of action of melaminyl arsenicals

As mentioned previously the primary site of action of organic arsenicals in mammalian cells is considered to be the α-ketoacid oxidases (Peters et al., 1946). Undoubtedly the toxicity to the mammalian host of these drugs is due to inactivation of metabolically active thiol groups, probably involved in oxidative reactions (Voegtlin et al., 1923; Johnstone, 1963).

From the known nature of the metabolism of monomorphic trypanosomes, the organic arsenicals would not be expected to exert any profound trypanocidal-effect. L.S. forms of T. brucei and T. rhodesiense do not oxidise pyruvate or α-oxoglutarate and cell lysates of these organisms do not have demonstrable pyruvate oxidase activity (Flynn and Bowman, 1973). In spite of this, there is a very rapid effect of melaminyl and other trivalent aromatic arsenicals on the flagellates, suggesting a site of action on the energy producing systems (Williamson, 1975). The site of action was found to be constant in monomorphic and pleomorphic lines of trypanosomes and also independent of α-oxoacid oxidases in vivo (Flynn and Bowman, 1973). Analyses of the mode of action of melarsen oxide, showed the terminal glycolytic enzyme (pyruvate kinase) of T. brucei to be the main focal target inhibited by the drug (Flynn and Bowman, 1968; 1969). A summary of the action of organic arsenicals, including melaminyl drugs, on semi-pure enzymes from members of the genus Trypanozoon is presented in Table 1.3.

The general mechanism of action of these toxic agents consists of at least two stages; a preliminary fixation, which may be reversible

and a subsequent lethal action at the target site (Clark, 1937). This idea correlates with the double functional feature of toxic agents proposed by Ehrlich in which the "haptophore" is involved in binding to a cell receptor and the "toxophore" exerts the lethal action. Melaminyl and other aromatic arsenicals are considered to have these characteristics, the organic residue being the haptophore and the arsenic moiety being the toxophore.

The selectivity of melaminyl drugs for the flagellates can not be explained in terms of thiol inactivation, as the equivalent target enzymes in the mammalian host are also inhibited (Flynn, 1971). Instead it is thought that this is controlled by the non-metallic portion of the molecule. Some effort has been put into the investigation of the effects of substituents on the organic residue to aromatic arsenicals (King and Strangeways, 1942). Lately it has been suggested (Newton, 1974) that changes in the substituent groups of organic arsenicals may alter the affinity of the compounds for their binding sites on enzymes or their ability to penetrate permeability barriers. The paramount importance of the organic residue is clearly recognised, not only in the uptake itself, but also in the degree of selectivity of the compound for the parasite. In the case of the melaminyl arsenicals the melamine ring serves to increase the preferential uptake of the drug by the parasite.

It is concluded that the rational approach to the development of resistance to melaminyl arsenicals should take into account this dual character ascribed to the drug. Therefore an analysis of the target enzymes and the mechanisms of uptake of melaminyl drugs was undertaken. The investigation was not confined to the study of isolated enzymes but also covered the study of the general metabolic properties of different lines of T. brucei with different degrees of sensitivity to melarsen oxide.

1.2.7 Arsenical estimation

The different methods available for the estimation of organic arsenicals can be broadly divided into bioassay techniques and physico-chemical estimation of arsenic after digestion of the organic matrix. Bioassay techniques have been used quite frequently in studies on drug resistance in trypanosomes (Yorke et al., 1931; Eagle and Magnuson, 1944). The procedure consists essentially in exposing a given number of parasites to a dilute drug solution, removal of the cells by centrifugation and determination of whether the drug concentration remaining in solution suffices to affect unexposed parasites. This routine has been adapted for the estimation of melaminyl arsenicals (Hawking, 1962), and primary information on drug resistance was developed with this technique. However this technique is unsuitable for estimation of the kinetics of the uptake of melaminyl arsenicals, which can only be studied with the help of a more accurate, sensitive and reproducible method for estimation of the drugs. It has been suggested recently (WHO, 1977), that for the development of the rationale behind drug resistance and also the general mechanism of drug action, radioactive drugs are needed. Unfortunately no labelled arsenicals were available at the time of this work.

By looking at the structure of a member of the melaminyl group, it can be seen that different potentially important methods can be used for estimation. Some routines have been tried; such as a modification of Marsh's test but this method relies on qualitative estimation (Hawking, 1963). Diazotization of the amino groups and coupling to a chromogen has been tried without successful results due to the instability of the melaminyl ring in acid conditions (Flynn, personal communication). Recently a new method of estimation of trypanocidal arsenical drugs, isotachophoresis, (Flynn, 1979) has been developed.

This technique was only developed after a chemical estimation method had been in use by the present author for a considerable time. The chemical method was therefore retained in order to have comparable results throughout the experimental work.

For the purpose of this work major attention has been paid to the estimation of arsenic in the drugs using physicochemical methods. Broadly there are three stages to consider:

1. Organic digestion of biological samples.
2. Recovery of arsenic from the digestion mixture.
3. Estimation of arsenic.

1. The treatment of the organic sample prior to analysis depends largely on the analytical method used for measurement of the arsenic (Lewis, 1977). For the assay of total arsenic content, wet digestion of the biological samples was chosen as a viable method. This method of ashing is accomplished through treatment of the material with strong mineral acids, peroxides or perchlorates. A small modification of the routine suggested in the U.S. Pharmacopeia, (1976) was used in the present work. Details of the method are found in Materials and Methods.

2. For the recovery of arsenic from the digestion mixture, different methods are available. One of the most utilised involves the conversion of the arsenic to a highly volatile hydride $As_2H_3$ followed by quantitative evolution into a trapping solution (Lewis, 1977).

3. Again several techniques have been described to measure the arsenic, most determinative methodology being based on detection of atomic species or coloured complexes prepared from arsine. The technique of choice for the present work involves the production of a highly coloured complex of arsenic (III) with silver diethyl dithiocarbamate (AgDDc) (Dal Cortivo et al., 1960). This colorimetric method is reliable and the costs are low. The only major disadvantage is that the digestion of the organic

sample, generation and trapping of the arsine in AgDDC with several samples, is a time consuming routine.

### 1.2.8 Resistance; Historical account and definition of the problem

Franke and Roehe (1905) discovered the phenomenon of drug resistance when working in Ehrlich's laboratory. Infected rodents treated with sub-effective doses of trypanocides developed a recurrent parasitemia after a short period of time; after the relapse, a second treatment with the drug proved to be ineffective. The new resistant character was stable in the population of trypanosomes. It was then realised that organisms under treatment with chemotherapeutic agents become resistant to the drug to which originally they were sensitive. Observations of this kind have been reported since the beginning of this century and nowadays it is a common aspect of the chemotherapy of African trypanosomiasis (WHO/FAO, 1979). Arsenical resistance was first developed *in vitro* by Browning (1907), who also found that there was a variability in the time required to generate resistance according to the arsenical used. The development of resistance *in vitro* is now an accepted routine to obtain drug fast trypanosomes to certain drugs (Schnitzer and Grunberg, 1957).

Yorke and Murgatroyd (1930) suggested that the term arsenical resistance was improperly used since general tolerance to all arsenicals is not developed. Instead resistance is developed only to a certain class of arsenicals. Also Williamson and Rollo (1959) developed a resistant line of trypanosomes to melarsen, which was cross-resistant to other unrelated non-metallic drugs, indicating that the resistance was not directly related to the metal moiety. In view of these findings, and the ones presented in this work, it is suggested that the

term "arsenical-resistance" should be rejected and replaced by a more comprehensive definition which includes the organic residue, as well as the metal part of the drug.

As suggested above it was found that the development of resistance to a particular drug was accompanied by resistance to closely related structures. More details on this particular topic are discussed in the section 2.2.3 of this introduction. It is important to notice at this stage that resistance to melaminyl arsenicals is an established concept in the chemotherapy of African trypanosomiasis. It was found by Rollo and Williamson (1951) that resistance to melarsen was possible and the development of resistance to this structure produced cross resistance to tryparsamide and pentamidine. The list of species which have been rendered resistant to arsenicals includes T. equiperdum, T. equinum, T. brucei, T. gambiense and T. lewisi. Studies on resistance to organo-arsenicals have been carried out with different lines of the parasites and with many organic arsenicals.

Many reviews have been published on protozoan drug resistance including the phenomenon related to trypanosomiasis (Schnitzer, 1932; Schnitzer and Grunberg, 1957; Bishop, 1959; 1962; Hawking, 1966; Williamson, 1962; 1970). None of these reviews is able to give a substantial indication of developments in the field with regard to the mechanism by which resistance arises.

In the meantime it is useful to adopt definitions of drug resistance in order to have a frame of reference in later discussion:

> "Resistance can be defined as a temporary or permanent loss of the initial sensitivity of the microorganisms to the effect of an active substance. It manifests itself after exposure to agents in vivo or in vitro" (Schnitzer and Grunberg, 1957).

"it can be defined as a temporary or permanent capacity of the cells and its progeny to remain viable or multiply under environmental conditions that would destroy or inhibit other cells." (Bryson and Szybalski, 1955).

"the sudden or gradual, permanent or transient loss of the originally present susceptibility to a chemical compound." (Lamy, 1967).

Two main suggestions are found in these definitions. Firstly the stability of the change in the populations of trypanosomes is taken into account, after exposure in vivo and in vitro. Secondly the resistant character can be obtained by different mechanisms which implies different origins of drug resistance.

The experimental evidence gathered so far has shown some basic patterns by which resistance can be recognised. For example a drug fast organism no longer responds to the largest possible doses in vivo, or to high multiples of the minimal active concentration in vitro. Coupled to that, it has been found that trypanosomes can retain these characteristics for long periods of time even through cyclical transmission by insect vectors (Schnitzer, 1955; Gray and Roberts, 1968).

Finally it is important to note that the rational approach to the study of the mechanism of action of trypanocides and hence, the development of drug resistance, should consider the molecular basis of the relationship between these structures and their receptors.

### 1.2.9 Origin and genetics of drug resistance

#### Origin

Two basic questions can be put forward regarding the ultimate nature of the change occurring when microorganisms become resistant to active agents. First, how is the resistant character passed from

one generation to another. Second, what are the mechanism by which the parasites acquire resistance to certain drugs, this second question is dealt with in section 2.2.5.

Opposing views have always existed on the relative importance of selection of mutants as opposed to phenotypic adaptation, to explain the origin of drug resistance. In some instances it is likely that both processes may be involved (Bishop, 1959; Schnitzer and Grunber, 1957; Peters, 1970).

A classification of the possible origins of drug resistance was offered by Bryson and Szybalsky (1955); a summary of the classification is presented as it resumes most of the discussion on this particular aspect:

A. Resistance primarily dependent upon genotypic change
    1. - Mutation
        1.1 - spontaneous
        1.2 - induced
    2. - Genetic exchange
        2.1 - by gametes
        2.2 - by transduction/transformation
B. Resistance dependent upon non-genetic change of phenotype
    1. - Introduction of a new physiological function
    2. - Elimination of cytoplasmic structures
    3. - Accumulation of drug inactivating factors
    4. - Selection of an alternative physiological function.
C. Resistance dependent upon composite changes.

Obviously the classification presented is completely operational and it can be seen that some subdivisions are not mutually exclusive.

In order to understand the two problems suggested at the beginning of this section, particularly regarding the heredity of any possible change in drug sensitivity, the cytogenetics of trypanosomes must be clarified. Little concrete information is available on this aspect of the life cycle of these parasites, as evidenced by a recent report on the life cycle of T. brucei (Ormerod, 1979).

The genetic aspect of drug resistance related to the biology of trypanosomes have been discussed many times (Schnitzer and Grunberg, 1957; Bishop, 1959; Walker, 1964) but no definitive conclusion has emerged.

Regarding the transfer of genetic material, there are opposed views; on one side, those suggesting the transference of the resistant character (Inoki and Matsushiro, 1960; Inoki et al., 1961) and on the other side, those who did not find any exchange of genetic material (Amrein, 1965; Hawking and Walker, 1966). In spite of this difficulty, development of drug resistance in terms of diploid expression and genetic exchange has been suggested by Walker (1964).

To conclude, it is suggested that resistance in a population of trypanosomes is acquired by a succession of genetic events that are favourably selected when the drug is present (Hawking and Walker, 1966).

1.2.10 Cross resistance studies

Resistance to trypanocidal compounds is not necessarily confined to the structure to which the resistant line has been developed; cross resistance can occur between compounds differing in chemical structure (Bishop, 1962). The cross resistance patterns in arsenical resistant lines of trypanosomes have resulted in a very complicated picture. Furthermore, arsenic resistance as such does not exist as organisms highly resistant to one group of arsenicals can retain their sensitivity to other arsenicals (Williamson and Lourie, 1948). This complex behaviour could be explained using King and Strangeways (1942) data. They postulated that there were different mechanism of uptake for different organic arsenicals.

Table 1.4: Cross-Resistance Analysis of Trypanocidal Drugs

| Drug group | Drug | Ionization at blood pH | Response of strain resistant to | | | |
|---|---|---|---|---|---|---|
| | | | Atoxyl | Melarsen | Butarsen | Suramin |
| Neutral aromatic arsenicals, antimonials | Atoxyl | Feeble | + | + | + | − |
| 2,8-Diaminoacridines | Acriflavine | | + | + | + | − |
| Melaminyl arsenicals, antimonials | Melarsen | | − | + | − | − |
| (a) Aliphatic and | (a) Synthalin | | − | + | − | − |
| (b) aromatic diguanidines, diamidines | (b) Stilbamidine | Cationic | − | + | − | − |
| | Surfen C | | − | − | − | − |
| 6-Aminoquinoline-type | Quinapyramine | | − | − | − | − |
| | Cinnoline 528 | | − | + | − | − |
| Carboxylated aromatic arsenicals | Butarsen | | − | − | + | − |
| | Suramin | Anionic | − | − | − | + |

Based on Table 7.3 of Williamson (1970).

Most of the information on cross resistance is reviewed by Williamson (1970). A summary of the relevant data is presented in Table 1.4. This classification is based on the ionic characteristics of the different drugs. A recent classification by Zakrzewski (1973) used different categories to group the different cases of drug resistance; selective cross resistance and non-reciprocal cross resistance. In general, knowledge of the earlier stages of trypanocidal action has been largely inferred from drug resistance studies (Williamson, 1962).

However, little information is available on the mechanisms and kinetics of drug uptake or on the mechanisms by which mutual interference occurs. Both these facets of the problem should be taken into account when dealing with the subject of resistance.

1.2.11 Experimental development of drug resistance

The principal methods used for the production of drug resistant parasites have been reviewed by Schnitzer and Grunberg (1957). Drug resistance in trypanosomes, once it has fully developed, is a relatively stable character. Drug resistance persists with some variation depending on the drug and the line of trypanosome used (Bishop, 1959). A summary, of the classification of the methods for producing drug resistant organisms, is as follows (Schnitzer and Grunberg, 1957):

A. Development of resistance in vitro

B. Development of resistance in vivo

1. By repeated exposure

1.1 - in one animal

1.2 - in serial passages

a) relapse method

b) short passage method

c) continuous exposure

1.3 - development of resistance under reduced host response

2. By single exposure.

It can be seen that there is a variety of techniques which can be employed for the development of resistant protozoa. The final choice of a particular technique or techniques will depend not only on the availability of the drug, but also, on the sensitivity of the trypanosomes to the chemotherapeutic agent and the availability and type of animals in which the resistant line is going to be developed. Another important aspect, when developing drug fast organisms, is the time for the appearance of stable resistance which has been found to be rather variable (Hawking, 1963).

From the classification presented, two main routines have been evolved for the development of resistance in protozoa; *in vitro* and *in vivo* techniques. It is clear that the *in vivo* techniques have received considerably more attention than the *in vitro* techniques. The main reason for this is the difficulty of keeping living protozoa, particularly African trypanosomes, in axenic conditions. It must be mentioned that in spite of this difficulty the method has been used to produce a tryparsamide resistant line of *T. gambiense* (von Brand et al., 1953).

The present status of the *in vitro* cultivation of animal infective African trypanosomes has been reviewed recently by Hirumi et al. (1979). As reported earlier in the Introduction, the technique evolved for the cultivation of *T. brucei* requires the presence of a fibroblast feeder layer. The potential of this technique in the study of the development of resistance *in vitro* remains to be evaluated.

As discussed above, and in spite of the difficulties of keeping *T. brucei* *in vitro*, exposure to melarsen oxide for short periods of time, in a simple buffer system, was assessed as a meansof developing resistance.

After an appraisal of the methods, the technique of choice was the short passage method, first used by Margulis (1910) and Morgenroth and Freund (1924). This system of developing drug fast trypanosomes is the most frequently used, particularly in experiments carried out in small rodents; The principle consists of the interruption of the influence which the drug treatment exerts on the parasites by removing them from the environment of the treated host to a new host before a significant reduction in the parasitemia has taken place (Schnitzer and Grunberg, 1957). This method has been used recently by Damper and Patton (1976a) to produce pentamidine resistant lines of T. brucei and T. rhodesiense. One of the most valuable characteristics of resistant lines prepared by the short passage method is the fact that they retain their immunological properties. Finally a line of T. rhodesiense resistant to the pentavalent arsenical, Melarsen, was developed by Rollo and Williamson (1951) using a similar method to the short passage method.

1.2.12 Mechanisms involved in the development of drug resistance

With regard to the mechanisms of development of resistance, different categories have been postulated (Schnitzer and Grunberg, 1957; Pollock, 1960). It is clear that these classifications are operational; on the other hand one mechanism can not be considered to be independent of the others. Some times the experimental evidence is inadequate to show clear differences between the categories. A general classification by the above authors is as follows:

a. Changes in metabolic functions so as to eliminate the steps susceptible to the drug.

b. Inactivation of the drug by a drug fast organism.

c. Target altered in such a way that its sensitivity to inhibition is decreased or overcome.

d. Alteration in permeability with a subsequent decrease in the penetration of the drug.

a. Changes in metabolic functions so as to eliminate or by-pass the steps susceptible to drug action.

The basic metabolic functions of African trypanosomes have been characterised (Bowman and Flynn, 1976). This information can be used to assess any possible differences found in resistant lines of trypanosomes when compared to their parent lines. Knowledge of the metabolic characteristics of resistant trypanosomes has still to be developed. One of the first reports on this subject (Reiner et al., 1932) showed no difference in the respiratory rate of parent and arsphenamine resistant line of T. equiperdum. Some more preliminary evidence can be found in the work of Von Brand et al. (1953). These data suggested rather inconclusively that these may be a minor difference in the carbohydrate metabolism of T. gambiense resistant to tryparsamide. In spite of the small differences found, the latter author concluded, as did Harvey (1949), that there was no substantial change in the metabolism of carbohydrates by trypanosomes resistant to arsenicals. This view is not shared by Williamson (1953a,b) who suggested that a strain of T. rhodesiense fully resistant to melarsen, differed markedly from the parent line with respect to the utilisation of some substrates. However, these results are contradictory, because they suggested the presence of lactate dehydrogenase in the parent organisms. It is now a well accepted observation that the African trypanosomes do not have this particular enzyme in their repertoire (Dixon, 1966).

In general no definitive evidence exists on the metabolism of carbohydrates in trypanosomes resistant to drugs of the melaminyl series. Therefore, a more comprehensive approach should be taken in the design of experiments concerning possible metabolic differences that may arise as a result of the development of resistance to these drugs.

b. Inactivation of the drug by a drug fast organism.

As stated previously organo-arsenicals owe their toxicity to a great affinity for thiol (SH) groups (Voegtlin et al., 1923). This observation suggested that resistant trypanosomes may have large stores of SH groups to detoxify organo-arsenicals (Voegtlin et al., 1924), implying that the accumulation of organo-arsenicals by resistant trypanosomes would be as great if not greater than that by normal parasites. This hypothesis has been tested by various investigators but not detectable difference was found in the thiol content of normal and resistant trypanosomes (Hawking, 1938; Harvey, 1948; this work).

An increase in the ability to enzymically detoxify a drug by conjugation, oxidation or cleavage may also account for melaminyl resistance in trypanosomes. A classical example to illustrate this point, although not in the area of pathogenic protozoa, is the development of the drug destroying enzyme, penicillinase, in penicillin-resistant forms of bacillus and staphylococci (Benveniste and Davies, 1973).

c. Target altered in such a way that its sensitivity to inhibition is decreased or overcome.

It is reasonable to visualise that certain configurational changes in the drug-susceptible enzymes as well as changes in their activities in trypanosomes undergoing acquisition of resistance, could explain the problem, in macromolecular terms (Sevag, 1955). Abnormal metabolism of the cell, abnormal population dynamics, etc. may be related to altered states of target enzymes in the resistant parasites.

Sevag (1946) suggested that enzymological changes in trypanosomes undergoing the acquisition of resistance to thiazine dyes, involve a configurational change of the enzyme targets with subsequent loss in the affinity for the dye. It has been mentioned in a previous chapter that the kinases of trypanosomes are particularly sensitive to the

action of melaminyl arsenicals and other arsenicals in general. It has also been suggested (Flynn, 1971) that the presence of hydrophobic amino acid residues around or near the active site of pyruvate kinase are important for the binding of aromatic organic arsenicals, including melarsen oxide. Thus a minor modification in the primary structure of the sensitive protein around the binding site of the lethal agent, making the binding less favourable, could explain decreased affinity and hence resistance at this particular level. An example in this context is the strain of Plasmodium berghei resistant to pyrimethamine which has developed a modified dihydrofolate reductase with higher specific activity and with less affinity for the lethal agent, when compared with the parent enzyme (Pinder, 1971).

Finally if an organism can achieve a substantially increased synthesis of a target enzyme, it stands a chance of overcoming the inhibition by an active substance. Nothing is known about the differential activity of target enzymes to organo-arsenicals in parent and resistant lines of T. brucei.

T. brucei depends on glycolysis for the generation of ATP. As discussed earlier the kinases, particularly pyruvate kinase, are the most sensitive targets of organo-arsenicals including the melaminyl series. In this work pyruvate kinase and phosphoglycerate kinase were studied in relation to their kinetics and sensitivities to melarsen oxide in both drug sensitive and resistant line.

d. Alteration in permeability with subsequent decrease in penetration of the drug

The evidence pertaining to this category is sometimes difficult to appraise, due in part to the different methods used to estimate the drug (especially arsenicals). It is clear that more accurate techniques for the estimation of these drugs are required to make the results of experiments on permeability more interpretable.

The experiments of Yorke et al. (1931) first showed that an arsinilic acid fast line of T. rhodesiense did not absorb as much drug from solutions as did the parent line. Hawking (1937) corroborated these findings in experiments with trivalent arsenicals and tartar emetic. His data indicated that resistant trypanosomes did not absorb the compounds to which they were resistant, but took up other arsenical compounds to which the strain was still sensitive. Similar conclusions were reached by Eagle and Magnusson (1944) using a spontaneously developed line of T. equiperdum. This type of evidence is not exclusive to organo-arsenicals, the same kind of observations having been made by Hawking (1934) and Fulton and Grant (1955) when working with acriflavine and stilbamidine, respectively. Other workers (Williamson and Rollo, 1959; Williamson, 1959, a,b) from their experiments on selective interference, cross resistance and the effect of metabolic inhibitors suggested that a stereospecific change associated with the initial drug uptake may occur in resistant trypanosomes. However, a change in permeability as a result of the acquisition of drug resistance does not seem to be a general rule as found by Ormerod (1952), who reported similar levels of drug uptake with the parent line, and a spontaneously developed line of T. equiperdum resistant to antrycide, stilbamidine and 2-hydroxystilbamidine. It has also been found that although pentamidine and stilbamidine resistance is associated with loss of the kinetoplast in T. brucei, this condition itself did not alter drug transport when developed above. However it was found that pentamidine has lower rates of transport in trypanosomes resistant to this drug (Damper and Patton, 1976,b). Similar results showing practically no transport of melarsen oxide into a resistant line of T. brucei are shown in this work.

On the basis of the evidence presented above it is clear that although something is known about the general mechanism of resistance,

explained in terms of differential permeability, a more detailed study of the mechanisms of transport of organo-arsenicals is required, taking into account the kinetics of transport. Such a study has not yet been reported for any organo-arsenical.

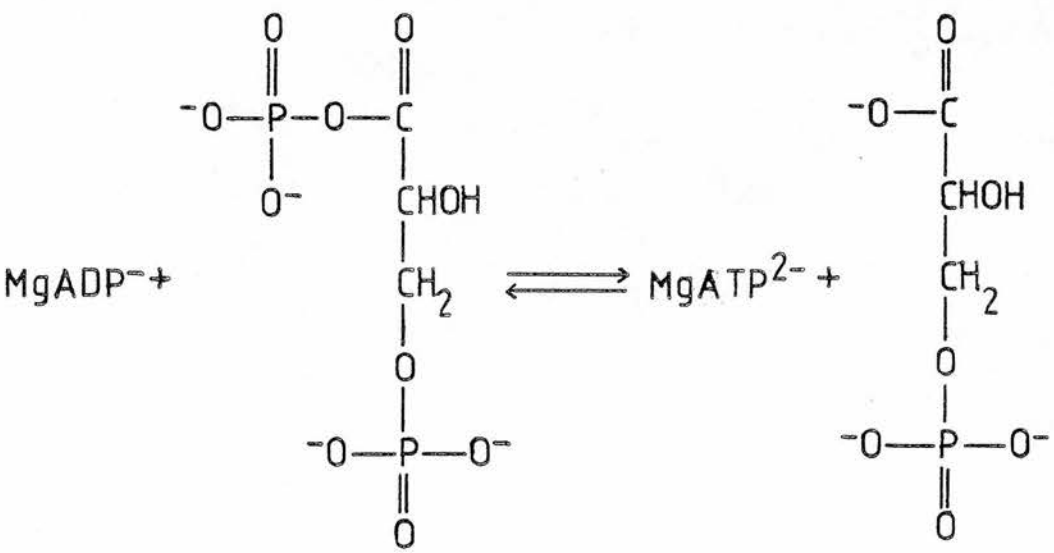
## 1.3 Phosphoglycerate kinase

### 1.3.1 General properties

It has been reported earlier in this thesis that the kinases are particularly sensitive to the action of arsenicals and indeed the mechanism of inhibition by melarsen oxide has been characterised for pyruvate kinase (Flynn, 1971). The action of other organic arsenicals on hexokinase and phosphofructokinase has also been reported (Chen, 1948; Jaffe et al., 1971). Phosphoglycerate kinase (ATP:3-phospho-D-glycerate-1-phosphotransferase; E.C. 2.7.2.3) was among the glycolytic kinases, the least characterised in trypanosomes. Therefore, a closer look was taken at this enzyme, regarding the general kinetic properties, the mechanism of action and the potential inhibition by arsenical drugs.

The introductory review of the properties of mammalian phosphoglycerate kinase is presented to enable comparison between the host and the trypanosome enzyme.

Phosphoglycerate kinase, hereafter PGK catalyses the transfer of high energy phosphate from the acid anhydride bond of 1.3 diphosphoglycerate to ADP (Larsson-Raznikiewicz,1964; 1967).



The biological occurrence of PGK has been studied recently by Fifis and Scopes (1978). Using affinity elution chromatography, they isolated the enzyme from a variety of sources and the structural and kinetics parameters showed that the enzyme was a highly conservative protein. PGK is a key enzyme in the glycolytic pathway of aerobes and anaerobes and in carbon fixation in plants. PGK from various sources has shown variation in the motility patterns, in gel electrophoresis (Scopes, 1968; Bendall, 1961; Engers and Madsen, 1968). Recently Wolfgang et al. (1979) showed the presence of multiple forms of PGK in skeletal muscle and erythrocytes using C.M. sepharose.

1.3.2 Isolation and physicochemical properties

The enzyme,from various sources, has been isolated in crystalline form (Bücher, 1947; Kriestsch and Bücher, 1970; Scopes, 1971; Blake et al., 1972). It has also been shown that it is a monomeric protein with two globular domains and with a single polypeptide chain, as suggested by x-ray diffraction analysis (Blake et al., 1972; Wendell et al., 1972). This structure is not specific for PGK but is similar in other kinases e.g. hexokinase and adenylate kinase (Anderson et al., 1979).

The specific activity of the pure enzyme from different sources is around $10^3$ units/mg protein for the back reaction (Scopes, 1969; Krietsch and Bücher, 1970). It has a molecular weight of between 45,000-50,000 depending on the technique used (Scopes, 1973). The amino acid composition of purified PGK from different sources is again highly conservative; lysine, aspartic acid, glycine, alanine, valine and leucine are the most abundant residues while histidine, tyrosine and tryptophan are the least abundant (Larsson-Raznikiewicz, 1970; Krietsch and Bücher, 1970; Yoshida and Watanabe, 1972). PGK cannot be dissociated into smaller subunits. It is formed, as reported above,

of a single amino acid chain with N-acetyl serine as the $NH_2$ terminal residue and isoleucine as the COOH terminal residue (Banks et al., 1979).

For the large scale purification of PGK, Cibachrom blue, a structural analogue of ATP has been used. Reproducible purification has been obtained from Sacharomyces cerevisiae (Kulbe and Schuer, 1979). As reported Fifis and Scopes (1978),using affinity chromatography, isolated the enzyme from twenty different sources.

1.3.3 Thiol content in phosphoglycerate kinase

Variable results have been observed with respect to the thiol content of PGK from different sources (Kriestsch and Bücher, 1972). Experiments with parachloromercuribenzoate (pCMB) and 55' dithiobis (2 nitrobenzoic acid) (DTNB) detected seven and one -SH groups in the purified enzyme from rabbit muscle and yeast respectively. It was also shown by these authors that $MgADP^-$ and 3 phosphoglycerate delayed the action of the thiol reagents on the rabbit enzyme, suggesting the involvement of -SH groups around the binding site of the enzyme. No inhibition was found in the yeast enzyme.

Similar results, with respect to the inhibition of activity with thiol reagents, were found using PGK from human erythrocytes (Choy and O'Sullivan, 1975). Nine out of the eleven -SH groups reported for the erythrocyte enzyme reacted with DTNB. Again thiol involvement around the active site was suggested by the delay in the inhibition by DTNB produced by $MgADP^-$.

The thiol character of trypanosomal enzymes is poorly understood, probably because of the difficulty in obtaining large quantities of a relatively pure enzyme for experiments on -SH group estimation and inactivation in the presence of substrates and thiol reagents. However, trypanosomal pyruvate kinase has been defined as a thiol enzyme not only because of its sensitivity to pCMB but also to different organic arsenicals (Flynn, 1971).

Finally as reported earlier in this Introduction, the kinases are particularly sensitive to the action of arsenicals in trypanosomes. Of these enzymes PGK is the least characterised. Some studies on the kinetics, mechanism of action and inhibition by organic arsenicals were therefore carried out on trypanosome PGK.

1.3.4 Kinetics and mechanism of action

The kinetic parameters of PGK have been evaluated (Bücher, 1947; Rao and Osper, 1961; Krietsch and Bücher, 1970). It was claimed from the data obtained for the backward reaction, that the mechanism proceeds according to the Michaelis-Menten model for both substrates. Typical Km values for the back reaction are: for $MgATP^{2}$ Km = 0.11-0.48 x $10^{-3}$M and for G3P Km = 0.63-1.36 x $10^{-3}$M. However, not all authors are in agreement with this, as is discussed below.

The nucleotide specificities of the yeast and muscle enzymes have been described (Krietsch and Bücher, 1970). In both cases the enzyme has the highest activity with ATP. However, ITP, GTP, dGTP and dATP supported activity when tested using the back reaction. As with other kinases, PGK has an essential requirement for a divalent cation for activity. $Mg^{2+}$ has been found to complex with either ADP or ATP; the complexes being the true substrates. Irregular results have however been found when working at high $Mg^{2+}$ concentrations (Larsson-Raznikiewicz, 1967). These data suggest that there are two binding sites for each substrate on the enzyme. Yoshida and Watanabe (1972) reported that the enzyme activity, as a function of the concentration of ATP, did not fit the Michaelis-Menten relationship. Again the suggestion was that two binding sites for the substrate were present. Several descriptions of the mechanism of action have been put forward suggesting the possibility of a rapid random mechanism for the binding of substrates (Larsson-Raznikiewicz and Arvidsson, 1971; Lee and O'Sullivan, 1975).

However, new evidence has suggested that the mechanism of action of yeast PGK is complex, and the concepts of substrate activation, double binding sites for $MgATP^{2-}$ and G3P, and product inhibition are gaining more ground (Schierbeck and Larsson-Raznikiewicz, 1979). Some disagreement seems to exist about the number of binding sites for G3P as suggested by Scopes (1978a;b), who advocates only one site for this substrate in yeast PGK.

As reported, the postulated mechanism of action of PGK obeys a rapid random reaction with no mutual interference for the binding of either substrate. Further evidence from initial velocity, product inhibition and substrate analogue inhibition studies, with $MgATP^{2-}$ as the phosphoryl donor, indicated that yeast PGK obeys this mechanism. Similar results have been obtained for other activating metals ($Mn^{2+}$, $Ca^{2+}$, $Co^{2+}$) and other nucleotides (ITP, GTP) with human erythrocyte PGK (Choy and O'Sullivan, 1975).

From the information derived from the crystal structure of the kinases it has been suggested that the enzyme possesses a deep cleft between the two domains of the protein. PGK is a good example of this group (Anderson et al., 1979), there is no definitive function for the feature. So far it has been found that the cleft is narrowed or closed upon binding of the substrates (Banks et al., 1979). These results have been corroborated by Pickover et al. (1979), using x-ray scattering of PGK in solution.

## 1.4 Final appraisal and objectives of the work

From the practical point of view, experiments concerning the mechanism of action of chemotherapeutic agents at the molecular level may generate, as suggested by Gutteridge (1978), new ideas which will eventually lead to the synthesis of new and more effective structures. The same argument could be used to rationalise the need for new research on the mechanism of development of resistance in trypanosomes, as the cause of the appearance of drug fast organisms is directly related to the chemotherapeutic agent and the way in which it produces its lethal action. Obviously there is a great interest in the mechanisms by which resistance can arise in trypanosomes, as this knowledge can help to develop methods of preventing the acquisition of resistance or of combating the resistance once it has arisen. A high priority has been given to this approach by WHO (1977) as it will not only contribute to chemotherapy but also to immunology and epidemiology of the disease.

For human trypanosomiasis four drugs are now available; suramin, nitrofurazone and melarsoprol (MelB) or melarsonyl potassium (MelW) used for curative purposes and pentamidine for chemoprophylaxis. No new drugs have been introduced for more than twenty years and all the drugs mentioned have serious side effects. The two organic arsenicals in use, Mel B and Mel W, are derived from the trivalent arsenical melarsen oxide. It is assumed that these complexes are catabolised *in vivo*, to the latter active structure. Therefore melarsen oxide was chosen as a model for the group of melaminyl arsenical drugs. It is on this structure that most of this thesis is based.

This work had the following main objectives:

1. To produce a fresh line of *T. brucei* resistant to melarsen oxide. Stabilates of different populations of trypanosomes exposed to different and consecutive doses of the drug, were prepared. The main aim was to

develop a Reference Collection for possible comparison among the lines obtained and possible extensions of the work.

2. To compare the basic metabolism of parent and resistant lines of T. brucei. It has been seen earlier in the Introduction that some information exists on the metabolism of arsenical resistant trypanosomes. However the information is inadequate and contradictory. Much information has been gathered on the glycolytic parameters of parent T. brucei. Hence, these characteristics are used as tools to compare the general metabolism of parent and resistant lines of the parasite.

3. To investigate the kinetics and the interaction of pyruvate kinase and phosphoglycerate kinase with organic arsenicals in parent and resistant lines of T. brucei. It has also been reported that the kinases are particularly sensitive to the action of arsenical drugs. Pyruvate kinase and phosphoglycerate kinase were considered key enzymes in the glycolytic sequence of these parasites, not only because of their potential sensitivity to the organic arsenicals but also because they are directly involved in the production of ATP at the substrate level. Therefore, the kinetic parameters and inhibition patterns of these enzymes, from parent and resistant lines, were investigated. As reported phosphoglycerate kinase is the least studied enzyme of the glycolytic kinase group in trypanosomes, therefore particular attention was given to the mechanism of action, mechanism of inhibition by arsenical drugs and other physicochemical properties of this enzyme.

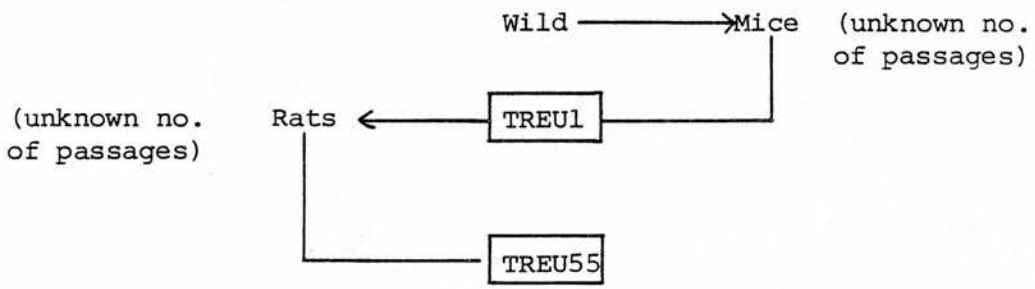
4. To investigate the uptake of organic arsenicals by parent and resistant lines of T. brucei. Finally, some of the information gathered on the uptake of arsenical drugs by resistant lines of parasites, points towards a reduced absorption of the drugs when compared to their parent lines. It is suggested that in this way the fast organism bypass the action of the active agent. Based on this assumption a closer look was taken at the mechanism of uptake of melarsen oxide and some other related arsenicals in parent and in a resistant line of the parasite.

# 2. MATERIALS AND METHODS

## 2. Materials and Methods

### 2.1 Line of T. brucei studied

The parent line of T. brucei TREU 55 (Trypanosoon Research, Edinburgh University) used in this thesis is a monomorphic population of trypanosomes derived from a wild population of T. brucei. The original sample of trypanosomes was maintained by serial passage in mice to give TREU 1 which was then subsequently passaged in rats producing TREU 55. The pedigree of the original parent line is as follows:




As mentioned above TREU 55 is completely monomorphic and it produces a fulminating parasitaemia with the subsequent death of the host animal 3 or 4 days after inoculation with the parasites.

### 2.2 Host and maintenance

Wistar albino male rats are used throughout the experimental part of this thesis as host animals for the T. brucei infection. For the establishment of infection, stabilates of T. brucei stored in 10% (v/v) glycerol at -70°C were allowed to thaw and diluted to give 15 to 25 motile organisms per microscope field when observed under high magnification (400X); 0.3 to 0.5 ml of the trypanosome suspension was inoculated intraperitoneally. The infection initiated in this fashion produced peak parasitaemia 72 hours later. For the further maintenance of the

infection, syringe passage was used every three days to infect clean animals. The procedure was as follows: infected blood from the tail of a parasited rat was diluted, with either citrated saline or phosphate saline (cf. 2.3), so that 10 to 15 parasites were visible per microscopic field under high magnification; approximately 0.3 ml of the trypanosome suspension was routinely injected intraperitoneally into each rat. Peak parasitaemia was again observed 72 hours after infection of the animals.

Stabilates were prepared as follows: infected blood was collected by exsanguination, after cardiac puncture of the right ventricle, into 1 ml of phosphate saline buffer with 5 IU/ml of heparin. The blood was diluted in PSG buffer (see Section 2.3) to give a final concentration of trypanosomes of approximately $5 \times 10^7$ cell per ml. The number of organisms was determined by haemocytometry. Glycerol was then added to a final concentration of 10% (v/v). The suspension was throughly mixed and dispensed immediately into plastic capped tubes (1.5 ml); after closing the tubes they were arranged in a small plastic rack and taken directly to a deep freeze at $-70^{\circ}C$. After a day the tubes were removed from the rack and placed in a plastic container in the same deep freeze. Stabilates prepared in this fashion kept the original infectivity even after seven months storage.

2.3 Buffer solutions

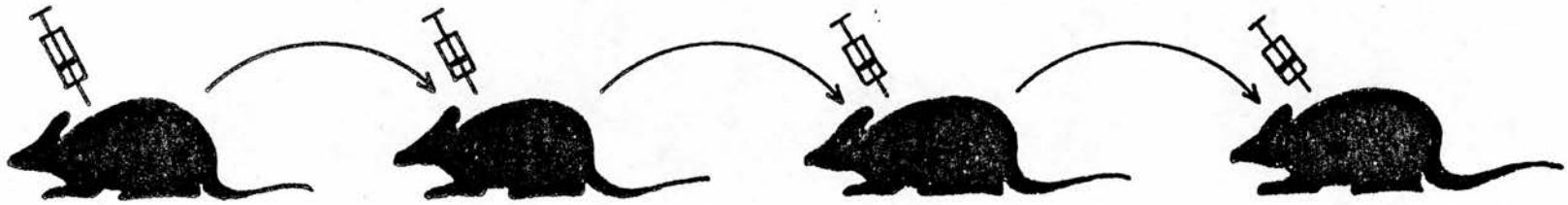
Phosphate saline (PS buffer): 53 mM disodium hydrogen phosphate, 3 mM sodium dihydrogen phosphate, 45 mM sodium chloride, pH 8.0

Phosphate saline glucose (PSG buffer): as PS buffer plus 1% glucose, pH 8.0.

Phosphate saline glucose albumin (PSGA buffer): as PSG buffer plus 0.5 mg/ml of bovine serum albumin (BSA), pH 8.0.

Tris-Sucrose: 10 mM Tris-HCl, 0.25M sucrose, pH 7.6.

Figure 2.1: The short passage method.



Wistar albino rats were infected intraperitoneally with trypanosomes as described in Section 2.2. Blood from the tail of the rats was monitored daily under phase contrast microscopy to estimate the number of circulating parasites. Once a high parasitaemia was reached the infected animals were treated with subeffective doses of melarsen oxide in phosphate saline. After therapy with the drug and before a considerable reduction in the parasitaemia has occurred, trypanosomes were used to infect a new group of host; the process was repeated with increased drug concentrations (see text for further details).

Tris-DTT: 100 mM Tris-HCl, 1 mM dithiothreitol, pH 7.5.

TEA: 10 mM TEA-HCl, pH 7.5.

Citrate saline: 157 mM sodium chloride, 51 mM tri-sodium citrate, pH 7.0.

## 2.4 Production of a resistant line of T. brucei using the short passage method

As discussed in the Introduction, there are different routines for the production of drug fast trypanosomes (Schnitzer and Grunberg, 1957). Two major routines have been used for the production of resistance in vivo: the relapse method and the short passage method; the first one has been discarded as it has been found that it produces serum resistant organism (Bishop, 1959).

The short passage method, first used by Margulis (1910) consists of the parenteral administration of a drug to experimentally infected animals with doses that approach the minimal or sub-effective drug concentration with continuous sub-passage of the treated trypanosomes. The main feature of the method is that the trypanosomes are removed from the treated host before the drug has exerted a considerable effect on the parasitaemia. As the number of passages increases, the dose to which the trypanosomes are exposed is also increased (see Figure 2.1).

This objective was accomplished in practice by infecting groups of 4 rats, using the already reported method for infection (Section 2.2). The animals were monitored for the development of parasitaemia by examination of a drop of blood from the tail. When the concentration of trypanosomes was approximately $10^8$ cell/ml the animals were weighed and inoculated with sub-effective doses of melarsen oxide. After therapy with the drug, the parasitaemia was further monitored and before a considerable reduction in the number of circulating parasites had occurred (10-20% reduction) a sample of parasites was used to infect a new group

of rats. The relapsing trypanosomes after inoculation were treated with higher concentrations of drug by repeating the procedure described above.

It should be mentioned that stabilates were produced throughout the development of the resistant character in order to produce a reference collection. Furthermore the development of the resistant character was monitored using a drug sensitivity test described in Section 2.13.

2.5 The isolation of trypanosomes from blood elements

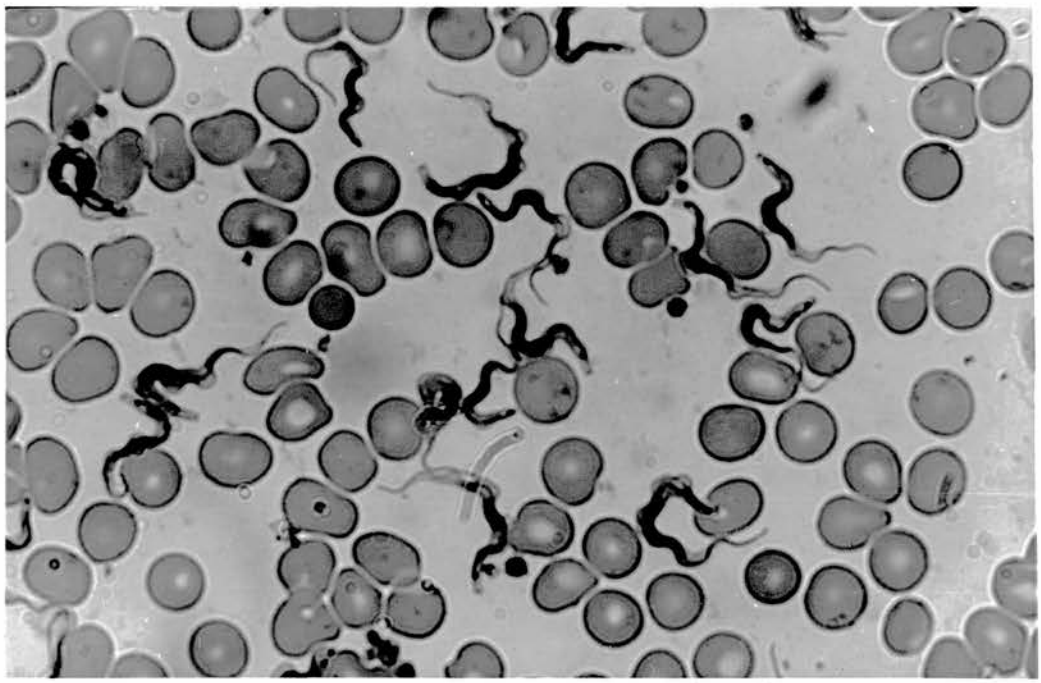
Trypanosomes were isolated from the bloodstream of infected rats when the parasitaemia was at its maximum, as indicated by inspection of blood from the tail. The method used was that of Lanham (1968). As reported by the same author the technique relies on the difference in charge at the surface of blood cells and trypanosomes. Isolation of the latter is carried out using an anion-exchange resin.

The procedure used was as follows: the blood from infected rats was collected, after cardiac puncture, into 1 ml of PS buffer containing 5 IU/ml of heparin. After centrifugation at 1000 g for 10 minutes at 4°C, the supernatant plasma was removed by aspiration and the white trypanosome layer was resuspended in cold PSG buffer; the centrifugation stage was repeated. The white trypanosome layer was pre-mixed with an equivalent volume of DEAE 52 previously equilibrated with PSG. The slurry was then poured on top of a DEAE 52 column (bed volume approx. 20 ml) and the trypanosomes were isolated from the remaining blood cells according to the method of Lanham (1968).

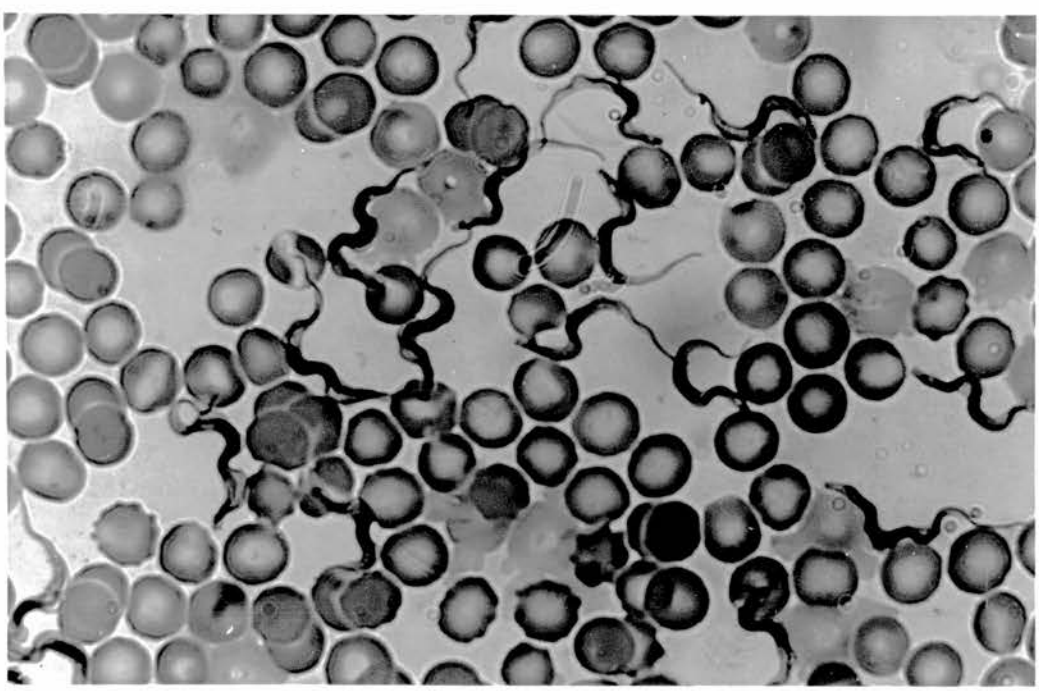
The method as described above produced a yield in terms of packed cell volume of approximately 0.6 to 1.0 ml per adult rat. To assess the motility and purity of the trypanosomes, prepared as described above, a sample of the final suspension of cells was examined under phase contrast microscopy. Only preparations with a 100% motile organisms, resuspended in the appropriate buffers according to the experiment performed, were used in this thesis.

Figure 2.2: The morphological appearance of the parent line and the resistant line of T. brucei.

Parent line



Resistant line



Stained blood smears were prepared from tail blood of infected animals with parent and resistant organisms. For details of the staining procedure and microphotography see Section 2.6.

## 2.6 The staining method and microphotography

Blood from the tail of infected animals was spread as a thin film on glass slides and allow to dry. The preparations were fixed with absolute methanol and allowed to stand for 30 seconds; the excess methanol was shaken off and the slides placed in Giemsa's stain for 50 to 60 minutes (1 volume of stain + 9 volumes of distilled water, pH 7.0). The slides were then removed from the stain and rinsed under running tap water and dried.

Stained trypanosome preparations were examined in a Vickers M17 microscope.

Microphotographs of slides prepared from the parent and resistant lines of T. brucei stabilate [BEU melarsen oxide 0.52] are shown in Figure 2.2. I am grateful to Dr. J. Haywood of the Biochemistry Department of the University of Edinburgh for taking the photographs shown in this thesis.

## 2.7 Trypanosome motility test

For routine evaluation of the quality of the trypanosome preparation, after isolation from blood elements, and for the purpose of some specific experiments described in this thesis, the trypanosome motility was qualitatively evaluated according to the following notation:

### Description of trypanosome activity

| Description: | Notation: |
|---|---|
| Most trypanosomes show maximal movement of body and flagellum | +++ |
| Most trypanosomes have frequent movement of body and flagellum but have become slow compared with above | ++ |
| Approximately half of the population have slow movement, the rest of the organisms have infrequent movement of body and flagellum | + |
| Most trypanosomes are moving with Brownian motion, except for infrequent movement of flagellum | +<br>o |
| No apparent movement is observed and there is a considerable number of lysed cells | o |

## 2.8 Preparation of trypanosome homogenates

### 2.8.1 For enzymological studies

A volume of packed cells prewashed in PS buffer was resuspended in 9 volumes of 2 times distilled water. The trypanosome suspension was then subjected to homogenisation by hand, with an ice cold teflon pestle in a glass homogeniser (Wesley Coe Ltd., Cambridge) in an ice bath. Approximately 20 strokes were necessary to produce a homogenate without whole cells, as indicated by observation under phase contrast microscopy.

The trypanosome protein solution, produced in this way, was then frozen in an ethanol bath at -40°C in a round bottomed flask and the protein was lyophilised. The dried material was stored in vacuo at 0 to 4°C.

### 2.8.2 For metabolic studies

0.5 ml packed cell volume (1000 g max, 10 minutes) of prewashed trypanosomes was resuspended in 30 ml of Minimal fortified medium, as described by Flynn and Bowman (1973) (details below); the process of homogenisation was done as above ensuring that the homogenate did not contain intact cells when examined under phase microscopy. The material prepared in this way was used immediately for metabolic studies (see Section 2.11).

The composition of the Minimal fortified medium was as follows: KCl, 200 µmoles; EDTA, 3 µmoles; nicotinamide, 25 µmoles; $MgSO_4$, 20 µmoles; BSA, 10 mg; phosphate buffer pH 7.4; 67 µmoles; ATP, 5 µmoles; NAD, 5 µmoles; total volume 3 ml (Flynn and Bowman, 1974).

## 2.9 The preparation of protein for enzymological studies

50 mg of freeze dried material (10 mg/ml) was resuspended in an appropriate buffer for the enzymological studies of PK and PGK (see Section 3.4.3). The suspension was hand homogenised using a Dounce homogeniser equipped with a tight fitting pestle; 20 strokes in an ice bath were necessary to produce a homogeneous suspension, as indicated

by examination under phase contrast microscopy. The homogenate was dispensed in plastic tubes and centrifuged for 1 hour at 100,000 g in a preparative centrifuge (Sorvall model L2-65). The rotor used was model 50 $t_i$ at fixed angle.

At the end of the centrifugation stage, the supernatant was poured on to a Sephadex G25 column of 20 ml bed volume, preequilibrated with the appropriate buffer. The protein peak was collected with the void volume and made 10% (v/v) with glycerol. The material prepared as above was used immediately for enzymological studies. A diagram of the procedure is found in Figure 3.16.

## 2.10 Enzyme assays

### 2.10.1 Pyruvate kinase

For the estimation of PK the method of Bücher and Pfleiderer (1962) was used and modified as suggested by Flynn (1971). The method depends on the coupling of pyruvate formation to lactate dehydrogenase producing lactate with the subsequent oxidation of NADH. One of the major advantages of this assay system is that the pyruvate is removed from the medium; therefore, the possibility of product inhibition is discarded with this system.

The assay principle to measure the activity of PK as suggested by Bücher and Pfleiderer (1962) is:

i) $PEP + ADP \xrightarrow{PK} pyruvate + ATP$

ii) $pyruvate + NADH + H^+ \xrightarrow{LDH} lactate + NAD^+$.

The components of the reaction mixture for the assay of trypanosome PK as suggested by Flynn (1971) are:

| Reaction mixture at pH 7.4: | Final concentration or activity: |
|---|---|
| TEA | $33.30 \times 10^{-3}$ M |
| $MgSO_4$ | $6.67 \times 10^{-3}$ M |
| KCl | $66.67 \times 10^{-3}$ M |
| NADH | $1.33 \times 10^{-3}$ M |
| LDH | 5 I.U. |
| ADP | $0.41 \times 10^{-3}$ M |
| PEP | $1.67 \times 10^{-3}$ M |

plus enzyme sample and water to 3 ml.

2.10.2 Phosphoglycerate kinase

The assay of PGK activity is often carried out using the back reaction of the enzyme leading from 3-phosphoglycerate to 1.3 diphosphoglycerate. The assay system used for the estimation of the activity of the trypanosome enzyme is based on the method originally described by Krietsch and Bücher (1970). The assay principle to measure the activity of PGK, as suggested by these authors is:

i) G3P + ATP $\underset{\longleftarrow}{\xrightarrow{PGK}}$ 1.3 diphosphoglycerate + ADP

ii) 1.3 diphosphoglycerate + NADH + $H^+$ $\underset{\longleftarrow}{\xrightarrow{GAP\text{-}DH}}$ 3 phosphoglyceraldehyde + $NAD^+$ + $P_i$

There is a known variation with regard to the cations and nucleotide requirements of PGK from different sources. Therefore it was necessary to study the particular requirements of the trypanosome enzyme; the experiments performed in this respect are shown in Section 3.41. Saturating concentration of all reactants in the assay system are used in order to ensure a representative activity of the enzyme. The concentrations were:

| Reaction mixture at pH 7.5: | Final concentration or activity: |
|---|---|
| TEA | $82.3 \times 10^{-3}$ M |
| $MgSO_4$ | $10.0 \times 10^{-3}$ M |
| EDTA | $0.96 \times 10^{-3}$ M |
| NADH | $0.16 \times 10^{-3}$ M |
| GAP-DH | 8.1 I.U. |
| ATP | $3.0 \times 10^{-3}$ M |
| G3P | $10.0 \times 10^{-3}$ M |

+ enzyme sample and water up to 3 ml.

Both enzymic assays involve the oxidation of NADH + $H^+$; therefore, the reaction can be followed spectrophotometrically at 340 nm. To this effect all the reactants in both enzyme assays were pipetted into silica cuvettes (1 cm light path) and preequilibrated in the spectrophotometer for not less than 5 minutes. The spectrophotometer used throughout the experiments was a Unicam model SP800 fitted with scale expansion and coupled to a Unicam slave recorder model AR25. The temperature of the cell compartment was 25°C maintained by means of a circulating water bath with pump.

The reactions in both assay systems were started by addition of the trypanosome enzyme, unless otherwise stated (e.g. inhibition studies). The initial velocities were estimated by tangential measurement to the trace on the slave recorder.

2.11 Standard manometric conditions

Purified trypanosomes, obtained by the method discussed in Section 2.5, were resuspended in PSGA buffer (4 mM glucose) after being washed twice in the same buffer. The final protein concentration was adjusted to approximately 0.8 mg/ml suspension.

The $O_2$ consumption was measured in a conventional Warburg instrument at 25°C with air as the gas phase; each flask contained

2.7 ml of trypanosome suspension with 0.1 ml of 40% (w/v) KOH absorbed on fluted filter paper, in the centre well of the flask, and 0.3 ml of 6M perchloric acid in the side arm. The flasks with the components described above or as otherwise stated were allowed to equilibrate for 10 minutes in the water bath of the instrument before starting the experiments. Since changes in the barometric pressure and minor fluctuations in the temperature are potential sources of error in the manometric readings, thermobarometers were submitted to the same experimental conditions as the other manometers and used to correct for the possible error mentioned above as described in Umbreit et al. (1957). The gas exchange was terminated by addition of perchloric acid from the side arm.

For the determination of the respiratory quotient (RQ) the simultaneous determination of $O_2$ consumption and carbon dioxide production ($CO_2$) is required. The Direct Method of Warburg was used for the determination of $CO_2$ in the presence of oxygen consumption (Umbreit et al., 1957). This method uses two identically prepared reaction flasks, the only difference being that one contains alkali in the centre well and hence the $O_2$ evolution can be determined; the other flask is prepared without alkali and therefore records the balance between $O_2$ consumption and $CO_2$ production. By taking in to account the readings in the respective manometers and their respective flask-manometer constants for oxygen and $CO_2$, it is possible to calculate the gas exchange and subsequently the RQ value (Umbreit et al., 1957).

The possibility of differential metabolism in the trypanosomes as a result of the different treatment of the preparation (with and without KOH) has been evaluated (Flynn, 1971) and no evidence has been presented to suggest that a particular metabolic change occurs attributable to

the presence of a $CO_2$ sink in one of the flasks. As mentioned in the metabolic section (see Section 3.2), there was a minor binding of atmospheric $CO_2$ due to the phosphate based buffer used (even when freshly prepared); correction was made for this binding by treating two thermobarometers, with equivalent volume of buffer, in the same way as the experimental manometers (with and without $CO_2$) and correcting for any $CO_2$ found in the buffer after acidification with perchloric acid.

Immediately after acidification and deproteinisation of the flasks contents, determination of metabolites was carried out. The procedure used was as follows: the flasks were emptied into chilled conical centrifuge tubes and the denatured protein removed by centrifugation. Aliquots of the supernatant are neutralised with 3M $KHCO_3$. Samples from the supernatant, after centrifugal removal of perchlorates as a potassium salt, were used for metabolite estimation (see next section).

For the determination of $O_2$ evolution in water lysates the procedure was exactly the same as for whole cells, except for the resuspension of the homogenate in a Minimal fortified medium to allow for gas exchange (Flynn and Bowman, 1973). Details of the preparation of the water lysate and the Minimal fortified medium are found in Section 2.8.2.

2.12 The estimation of metabolites

After deproteinisation of the flask contents at the end of the incubation period, metabolite consumption and production was measured enzymically using the method of "end point estimation" (Bergmeyer, 1978). Other metabolites closely related to the metabolism of carbohydrates in trypanosome are glucose, pyruvate and glycerol; they were measured using the method suggested by Fairlamb and Bowman (1980) in a single procedure. The method is as follows: deproteinised samples (0.06 ml) were assayed in a reaction mixture containing 50 x $10^{-3}$M Tris HCl,

pH 7.5 with 50 x $10^{-3}$M KCl, 10 x $10^{-3}$M $MgSO_4$, 0.2 x $10^{-3}$M EDTA, 0.15 x $10^{-3}$M NADH, 40 x $10^{-3}$M ATP and 50 x $10^{-3}$ phosphoenol pyruvate; with distilled water to a total volume of 3 ml. The pyruvate concentration was measured after addition of lactate dehydrogenase (5.0 IU/ml) as a decrease in the absorbance at 340 nm. Pyruvate kinase was added (4.0 IU/ml) and after a new steady absorbance was obtained glycerol kinase was added (1.7 IU/ml) to measure glycerol concentration, followed by hexokinase (1.4 IU/ml) to measure glucose concentration. The absolute concentrations of these metabolites were determined using standards that have been treated as the unknown samples.

For the studies on carbon balance from glucose, samples were treated as in Section 2.11, except that a zero time sample was used to define the substrate changes over any period of incubation chosen.

2.13 Drug sensitivity tests with the oxygen electrode

Trypanosomes, isolated as described in Section 2.5 were used for a routine drug sensitivity test; the cells were resuspended in PSG buffer to a final concentration of approximately 0.8 mg of trypanosome protein/ml, and incubated with different concentrations of melarsen oxide (range 0.1 x $10^{-6}$M-9.0 x $10^{-6}$M) in a Clark type oxygen electrode (Yellow Spring Instruments Co.) polarised at -0.8V. The electrode was adapted to fit a perpex reaction chamber of 3 ml capacity, containing a magnetic stirrer. The system was connected to a Servoscribe potentiometric recorder. The incubation chamber was thermostatically regulated with a water jacket connected to a water bath with pump set at 37°C. For the calibration of the oxygen electrode the value of 2.17 x $10^{-6}$ $O_2$ in air saturated water was used for calculations (Kielley, 1963); for the setting of the base line in the absence of oxygen, sodium dithionite was added to the water in the chamber and allowed to equilibrate.

The linear initial rates of oxygen consumption (nmoles $O_2$.$min^{-1}$.mg $protein^{-1}$) by trypanosome suspensions, at different concentrations of melarsen oxide, are compared with untreated trypanosome suspensions and presented as percentages. Duplicate values were usually obtained at each concentration of drug determined in palindromic series.

The system was used during the production of the resistant line; a typical set of inhibition patterns is shown in Figure 3.2.

2.14 The preparation of glycosomes and estimation of multienzyme activity

Trypanosomes isolated from blood elements as described in Section 2.5 were homogenised with the prewashed abrasive, silicon carbide (Norton Company, grit no. 37C 400). 1.5 ml of packed cell volume was treated with approximately 7.5 g of the abrasive and ground in a chilled mortar until no intact organisms were observed when examined by phase contrast microscopy. The homogenate and the abrasive were resuspended in enough Tris-sucrose buffer to allow transference of the mixture to 50 ml plastic tubes and centrifuged for 5 minutes at 100 g (max) in a MSE Mistral 4L centrifuge using a 8 x 50 ml swing out rotor. After centrifugation the supernatant was carefully decanted, the pellet resuspended and the process repeated. The combined supernatants, free of silicon carbide, were kept on ice and used as soon as possible for the purification of glycosomes.

For the purification of glycosomes the method of Oduro (1977) was used. The suspensions from the grinding with silicon carbide was centrifuged for 20 minutes at 1000 g (max) in a MSE Mistral 4L centrifuge at $4^{\circ}C$ in a 8 x 50 ml swing out rotor. The supernatant was collected and the pellet resuspended in Tris-sucrose buffer. Centrifugation and washing were repeated twice, and the supernatants saved for further purification.

The combined supernatants were centrifuged for 20 minutes at 14,500 g (max) in a MSE 18 centrifuge at 4°C in a 8 x 50 ml fixed angle rotor. The supernatant was discarded and the pellets resuspended in Tris-sucrose buffer; the centrifugation and washing step were repeated twice. The final pellet was resuspended in Tris-sucrose buffer and used for the estimation of glycosome multienzyme activity.

The multienzyme activity of the glycosome was estimated using the method of Oduro (1977). The assay system relies on the catalytic activity of the glycosome, from glucose to triose phosphates and then to G3P. The reaction catalysed by glyceraldehyde phosphate dehydrogenase is blocked by iodoacetic acid; multienzyme activity is followed by the oxidation of NADH with the reduction of DHAP to glycerol-3-phosphate, by glycerolphosphate dehydrogenase.

The components of the assay medium were as described by Oduro et al. (1980): TEA-KOH, pH 7.6, 97 x $10^{-3}$M; ATP, 0.94 x $10^{-3}$M; $MgCl_2$ 13.9 x $10^{-3}$M; NADH, 0.18 x $10^{-3}$M; iodoacetate, 1 x $10^{-3}$M; sucrose, 0.25 M; glucose, 400 x $10^{-3}$M and enzyme, in a final volume of 3.0 ml. The reaction was initiated by the addition of glucose, after incubation for 5 minutes at 25°C. The reaction was followed spectrophotometrically using a Unicam SP800 with a slave recorder Unicam AR25.

2.15 Silicon layer filtration technique

Filtration through silicone layers has been used to study the translocation of different substrates in mitochondria (Halstrap and McGivan, 1979). The technique involves incubation with the substrate to be transported, followed by rapid termination of the incubation by filtering the particulate material through a silicone layer of a specific density. The technique has been used recently for studies on the transport of pentamidine in T. brucei (Damper and Patton, 1976a,b). A modification of the silicone layer technique as proposed by the latter

authors was used in this thesis in the studies on the transport of melarsen oxide and other arsenical drugs.

Conical microcentrifuge tubes (1.5 ml) were filled with three different layers: a bottom layer of 0.1 ml of perchloric acid, 6M; an intermediate layer of 0.1 ml of silicone oil, prepared beforehand by mixing 1 volume of silicone oil AR20 and two volumes of silicone oil AP100 (Wacker Co.), and a top layer of 0.5 ml trypanosome suspension in the exposing solution carefully loaded by the side of the microcentrifuge plastic tube. This system with perchloric acid and silicone oil is referred to as the silicone sandwich.

2.16 The incubation of trypanosomes for uptake studies

Trypanosomes isolated from blood elements, as described in Section 2.5, were resuspended in PSGA buffer to a final concentration of 5.0 mg of trypanosome protein per ml of buffer (about $10^8$ cell/ml). For the incubation of the cells, an $O_2$ electrode perspex chamber (3 ml) connected to a magnetic stirrer was used. The chamber was thermostatically regulated with a waterjacket and connected to a variable temperature waterbath with pump, which allowed the incubation of the cells at different temperatures.

The suspension of trypanosomes (1.35 ml) was placed into the chamber and allowed to equilibrate at the desired temperature; the drug was then added in solution (0.15 ml) and the suspension incubated. Triplicate aliquots (0.5 ml) were dispensed using an automatic pipette on top of silicone sandwiches and centrifuged after sixty seconds incubation, in a Beckman microcentrifuge at the maximum speed (equivalent 10,000 g). Upon contact of the trypanosomes with the perchloric acid layer the protein denatures and two phases appear; an acid soluble and insoluble layer. Details of the experimental conditions were chosen from the results presented in Section 3.6. The different layers obtained were then ready for estimation of the drug used.

In the case of time course uptake experiments, four microcentrifuges were used. Every 15 seconds after the addition of the drug, aliquots (0.5 ml) were dispensed using an automatic pipette onto the silicone sandwiches and centrifuged for 60 seconds. The experiments were repeated three times in order to obtain reliable results.

2.17 Determination of extracellular volume

One minor disadvantage of the silicone layer filtration technique is the extracellular fluid which passes through the silicone layer into the perchloric acid layer when the trypanosomes are centrifuged (Damper and Patton, 1976a). The total volume involved was calculated by labelling the exposure solution with $^{14}C$ inulin to a final concentration of $2 \times 10^5$ cpm/ml. The radioactive tracer ($3 \times 10^5$ cpm/10 µl) was added to 1.5 ml of trypanosome suspension (with 0.15 ml of blank solution) and after incubation and centrifugation, as described in the last section, samples of the perchloric acid soluble layer (0.1 ml) were estimated for radioactivity. No radioactivity was found in the acid insoluble layer.

As the original source of $^{14}C$ inulin had been stored without use for more than a year a previous purification of the material was carried out by desalting on a column of Sephadex G25 (approximate bed volume 10 ml). The high molecular weight material collected with the void volume was used for the experiments in this thesis.

2.18 Radioactive counting

The experiments on the estimation of $^{14}C$ inulin were the only protocols in this thesis using a radioactive compound. Aliquots from the perchloric acid layer were mixed with 10 ml of scintillation fluid: 0.02% (w/v), 1,4-bis-(5-phenyloxazol-2yl)benzene, 0.4% (w/v) 2,5-diphenyloxazole in 33% (v/v) Triton X100 and 67% (v/v) toluene. The radioactive counting was performed in a Packard model 3330 Tricarb

scintillation counter. As the perchloric acid produced quenching, correction was made by counting $^{14}C$ inulin standards with and without the acid.

2.19 The estimation of arsenical drugs

The drugs were estimated by chemical assay of the arsenic moiety; the routine used in this thesis is the one presented in the U.S. Pharmacopeia (1975). The method is divided into two main parts: organic digestion and arsine generation with subsequent trapping in AgDDC solution (BDH Chemicals Ltd.).

Samples containing drugs were placed in 125 ml conical flasks, 5 ml of $H_2SO_4$ (conc.) was added and the organic matrix oxidised by placing the systems on a hot plate. To ensure oxidation, $H_2O_2$ (30% v/v) was added dropwise until a colourless solution was obtained. The flasks and their contents were allowed to cool and 10 ml of distilled water were added and reheated until fumes of sulphur trioxide are evolved. The flasks were removed from the hot plate and again allowed to cool, 35 ml of distilled water were added rinsing the sides of the flasks.

For the generation of arsine, 10 ml of $H_2SO_4$ (20% v/v) were added to the flasks with 2 ml of freshly prepared potassium iodide solution (15% (w/v) in water) and 0.5 ml of stannous chloride (40% w/v in HCl conc.). The contents were mixed and allowed to stand for 30 minutes. At this stage the flasks and their contents are called generator systems.

While the incubation is being completed, 2 ml aliquots of silver diethyldithiocarbamate (AgDDC) in pyridine (0.5% w/v) were dispensed into small diameter test tubes. At the end of the incubation period, zinc (3 g) was added to the generator systems which were connected by means of a glass delivery tube, with plugs of glass wool impregnated with lead acetate, to the test tubes with the AgDDC solution; the gases evolved were allowed to bubble through the solution for at least 45 minutes. At the end of this period, these generator systems with trivalent arsenicals produced

arsine which reacted with the AgDDC ($AgS.CS.N(C_2H_5)_2$) to form a soluble red complex (Vogel, 1978).

Standard concentrations of drugs and appropriate blanks were submitted to exactly the same conditions as the experimental flasks and after preparation of the standard curves, by reading the absorbance at 525 nm, the concentration of the unknowns were estimated by interpolation in the calibration curves according to the extent of the colour development. Typical calibration curves for melarsen oxide and sodium melarsen are shown in Figure 3.33.

2.20 The estimation of proteins

Protein estimation was done either by the method of Lowry et al. (1951) or Bradford (1976). Bovine serum albumin (BSA) was used as the standard protein with both methods; the calibration curves were linear from 0-150 µg BSA with the first method and from 0-100 µg BSA with the second method. TEA buffer interferes with the method of Lowry et al. (1951) but not with the other method; therefore the method of Bradford (1976) was used accordingly to avoid interference with the protein estimation.

2.21 The estimation of protein thiol groups

The method of Ellman (1959) was used for the determination of -SH groups in the trypanosome protein; these groups exhibit variable reactivity towards the Ellman reagent 5'5'-dithiobis(2-nitrobenzoic acid) (DTNB) owing to steric factors and hence determination of total -SH groups requires the unfolding of the protein (Habeeb, 1972). The routine suggested by the latter author was used for the estimation of total -SH groups in denatured trypanosome protein and available thiol in native protein.

For the estimation of total thiol an aliquot of 0.1 ml of DTNB solution (40 mg DTNB in 10 ml of 0.1M sodium phosphate buffer, pH 8.0)

was added to 3 ml of trypanosome protein solution (in 2% sodium dodecyl sulphate (SDS), 0.08M sodium phosphate buffer, pH 8.0 and 0.5 mg/ml EDTA). The colour is developed for 15 minutes and absorbance read at 410 nm against protein solution in SDS to give apparent absorbance. For calculation of -SH group content, the net absorbance is employed with a molar extinction value of $13,600 M^{-1} cm^{-1}$. The protein concentration was estimated by the method of Lowry et al. (1951) in the original solution of protein.

For the determination of available thiol the reaction is performed as described above but in the absence of SDS; the absorbance is again read at 412 nm and the -SH content calculated in the same manner.

2.22 The preparation of organic arsenicals

Melarsen oxide and phenyl arsenoxide are insoluble in water, hence these drugs were routinely solubilised in a small volume (0.1-0.2 ml) of 40% (w/v) KOH, the alkaline solution was diluted to the required volume and titrated with 5N HCl. As suggested by Flynn (1971) the solution was titrated immediately to neutrality, otherwise a cloudy solution appears on back titration to neutral pH values. Possibly this is due to instability of the melaminyl ring at basic pH conditions.

2.23 Chemicals

All chemicals were of the highest purity available ("Analar" grade or its equivalent), obtained from BDH Chemicals Ltd. or Sigma Chemicals Co. Ltd.

Enzyme cofactors and substrates were obtained from Boehringer and Sohne Gmtl, Mannheim; glucose was obtained from BDH Chemicals Ltd. and bovine serum albumin from Armour Pharmaceutical Co. Ltd., Eastbourne.

Radioactive $^{14}C$ inulin was obtained from the Radiochemical Centre, Amersham.

Silicon carbide was a gift from Norton Abrasives Ltd., Welwyn, Garden City, Herts. The silicone oils were a gift from Dr. Steve Shears of the Biochemistry Department of the University of Edinburgh, obtained originally from Wacker Co. The trivalent and pentavalent arsenicals used in this work were a gift from May and Baker Ltd., Dagenham.

Dithiothreitol was obtained from Koch Light Laboratories, Bucks.

Sephadex G25 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

2.24 Statistical methods

The mean and the standard deviations of the mean were calculated using a texas SR-51-II calculator employing the following formulae:

$$\text{mean, } \bar{X} = \frac{x}{n}$$

$$\text{standard deviation. SD} = \sqrt{\frac{\Sigma(x-\bar{x})^2}{n-1}}$$

$$\text{standard error of the mean} = \sqrt{\frac{\Sigma(x-\bar{x})^2}{(n-1)\,n}}$$

Unweighted linear regression analysis of linear data were carried out on a texas SR-51-II calculator.

The probit transformation used to analyse the results from drug sensitivity test experiments in this work has been used to study toxicologic and biological assay data where quantal response is involved (Mather, 1964). Indeed the method was used to study the development of resistance to tryparsamide by Hawking and Walker (1966). In the present work the linear rates of oxygen consumption obtained from the drug sensitivity test are used for the probit transformation (see Section 3.13). The values expressed as the percentage of inhibition of the untreated trypanosomes are converted into probit number using the tables already prepared for this purpose (Fisher and Yates, 1963). A plot of the logarithm of the

concentration of drug against the probit number gives a straight line, which represents in our case an approach to the description of the normal distribution of drug sensitivity of a determined population of organisms. As discussed by Hawking and Walker (1966), the straight line should represent a homogeneous population of individuals with a "normal" distribution of sensitivity to a particular agent. However if non-linearity is obtained it could be inferred that a heterogeneous population exists with respect to the sensitivity to the active agent, as at this point the plateau seems to represent the number of organisms which survive a particular range of drug concentrations. Although further conclusions could be obtained from this type of mathematical analysis, only linear traces were observed at less than 40% inhibition. Therefore the plots are used as a semiquantitative description of the possible development of a heterogeneous population with respect to the sensitivity to melarsen oxide.

2.25 The treatment of kinetic data

The kinetic data was analysed using an unweighted linear regression of the Hanes plot (s/v vs S). In some cases it was also necessary to use the Lineweaver-Burk plot (1/v vs 1/s) for comparative purposes, as it was found that most of the analysis of the mechanisms of action of PGK from other sources was carried out with this plot.

In other cases where the reaction with respect to a substrate was apparently different from first order, after linear transformation, the Hill plot was used ($\log \frac{V}{V_{max}}$ vs log S); the estimate of the $V_{max}$ was obtained from the Hanes plot. The Hill plot was mainly used to describe the phenomenon of negative cooperativity (Levitzki and Koshland, 1969) at low concentration of substrates with trypanosome PGK.

Where possible, two non-linear regression computer programmes were used: one to estimate the Michaelis-Menten parameters (Atkins and

Gardner, 1977) and the other to estimate the parameters of the Hill equation (Atkins, 1973). The Edinburgh University EMAS computer was used for this purpose. My thanks are due to Dr. G. Atkins of the Biochemistry Department of the University of Edinburgh, for the preparation of the programmes and guidance when using the computer.

To study the reaction velocities under the influence of temperature, the Arrhenius equation was used ($\ln k = \ln A - (Ea/Rt)$). The plot of log k (rate of reaction) against the reciprocal of the absolute temperature gives a straight line of slope $-Ea/R \times 2.3$ from which the energy of activation (Ea) can be calculated.

The thermodynamic parameters corresponding to the transition state; enthalpy of activation ($\Delta H^{+}$), entropy of activation ($\Delta S^{+}$) and Gibbs free energy ($\Delta G^{+}$) at 300 k were estimated using the following equations:

$$\Delta H^{+} = Ea - RT$$

$$\Delta S^{+} = R \ln (ANh/RT) - R$$

$$\Delta G^{+} = \Delta H^{+} - T\Delta S^{+}$$

where R = gas constant; T = absolute temperature; N = Avogrado's number; h = Planks constant and A = constant calculated from the Arrhenius plot.

For the treatment of data from inhibition experiments the methods of Dixon (1953) and Hunter and Downs (1945) were used. The method of Dixon (1953) allows the estimation of $K_i$ if the rate of the reaction is determined with a series of inhibitor concentrations keeping the substrate concentrations constant. A plot of the reciprocal of the initial velocity at different concentrations of inhibitor and at different fixed concentrations of substrate gives straight lines which are used to determine the constant of inhibition ($K_i$), according to the type of inhibition found (Dixon and Webb, 1964).

In some instances the protocol allowed the use of the method of Hunter and Downs (1945), in which the following equation is used:

$$i \frac{V_1}{V-V_1} = K_1 + \frac{K_1}{K_m} \cdot S$$

where v = initial velocity; $V_1$ = initial velocity with inhibitor (i) at specific substrates concentrations (S). The plot of S vs $\frac{V_1}{V-V_1}$ gives a straight line of intersection $K_1$.

Details of the derivation of the two methods used to study inhibitory constants are found in Dixon and Webb (1964).

# 3. RESULTS

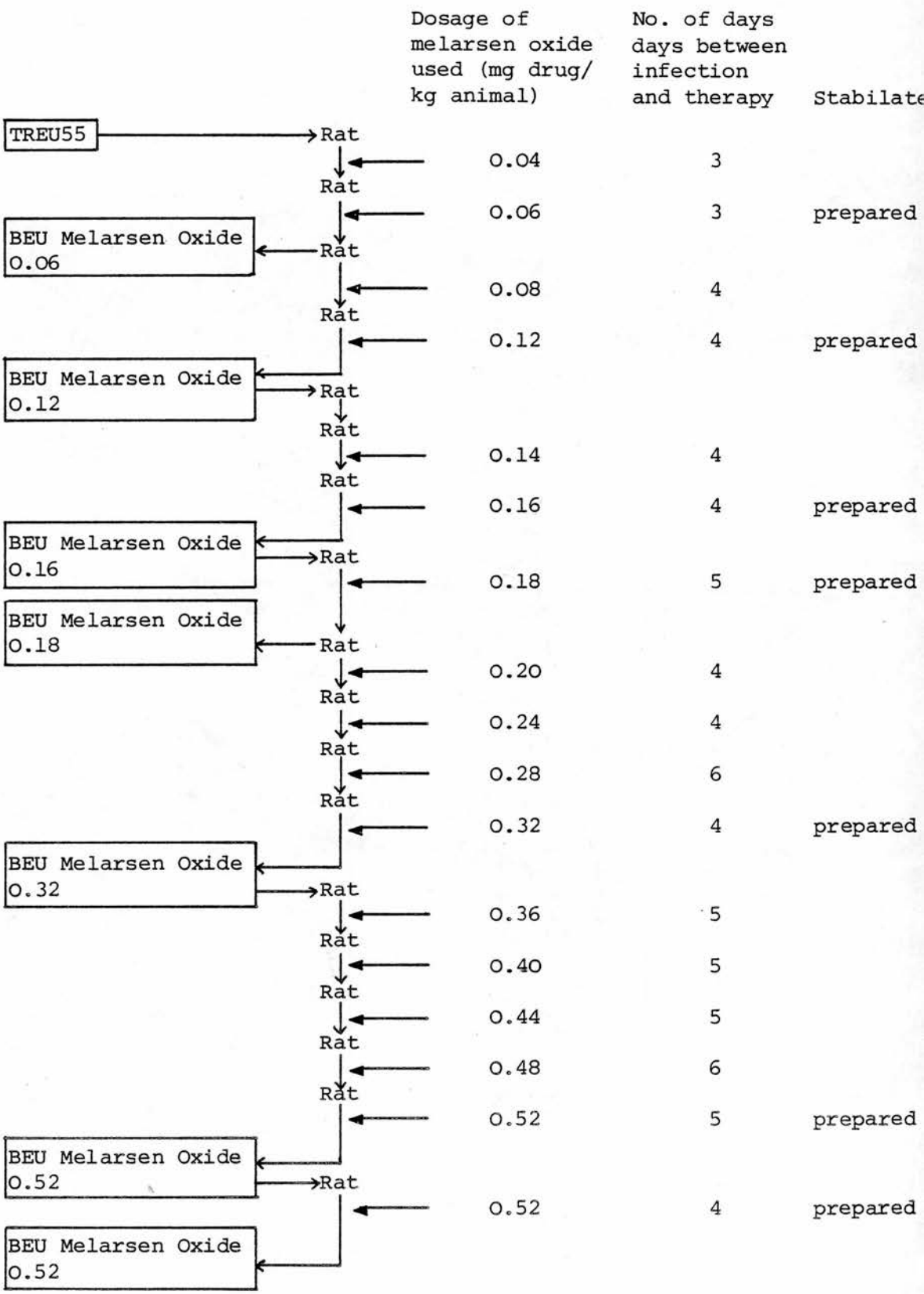
## 3.1 The production of the resistant line

### 3.1.1 The development and pedigree of the resistant line

Different methods were evaluated for the production of a resistant line of T. brucei. The first of the routines tried was the short in vitro exposure of trypanosomes, isolated by the method of Lanham (1968), to phosphate glucose buffer (PSG) containing different concentrations of melarsen oxide. The range of concentrations varied from $10^{-6}$ to $10^{-3}$M melarsen oxide. After checking by phase contrast microscopy to ensure survival, the trypanosomes were injected into rats and the potential development of parasitaemia was followed. This approach was tried on three occasions, and each time no trypanosomes were recovered even after a period of 15 days. The method was abandoned, and a second routine was used. Trypanosomes were injected into healthy rats, and once a high parasitaemia was reached (approximately 50 to 100 cells per microscope field at high magnification), the infected rats were inoculated intraperitoneally with melarsen oxide to produce a temporary clearance of the parasites from the bloodstream. A starting dose of 0.08 mg/kg was used (Rollo et al., 1949); however this dose produced total clearance for at least 15 days so it was reduced in order to obtain faster relapses. The results obtained with this method were irreproducible and also there was always the uncertainty of whether the trypanosomes were going to relapse after a certain critical dose. The method was abandoned due to these disadvantages.

The other two principal methods for the production of drug fast lines in vivo are the relapse method and the short passage method (Schnitzer and Grünberg, 1957). The first was rejected as it produces serum resistant organisms at the same time as resistance develops (Bishop, 1959), and because it has been suggested that it takes longer to develop resistance to arsenicals (Yorke et al., 1932). The alternative routine, the short passage method (as described in Materials and Methods)

Figure 3.1: The pedigree of stabilate [BEU Melarsen oxide 0.52].



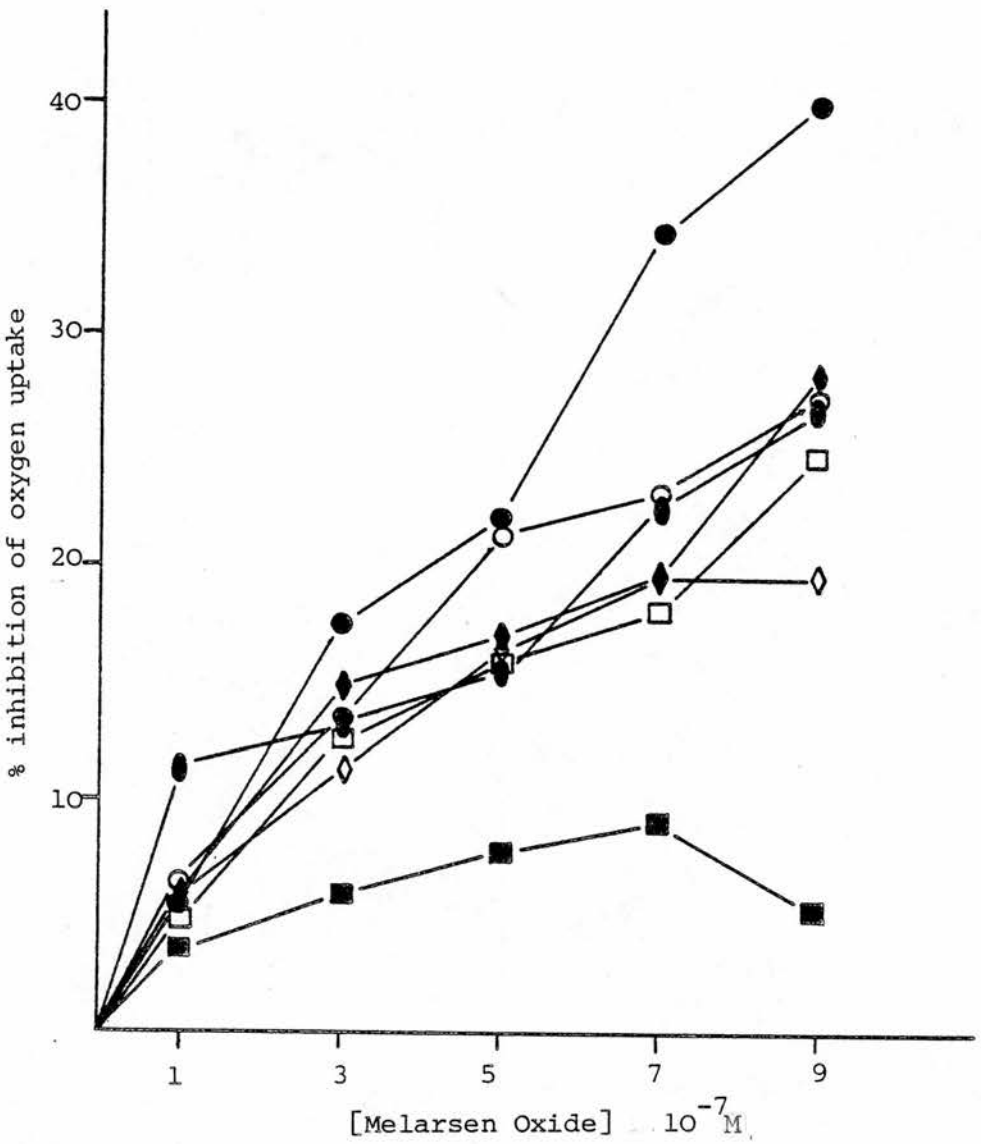

A reference collection was obtained of stabilates during the production of a melarsen oxide resistant line of T. brucei, originally sensitive to the chemotherapeutic agent. Detail of the nomenclature used in the pedigree appears in the text. The short passage method was used for th production of the resistant line; for details see Materials and Method (Section 2.4).

was the final method of choice. The starting dose used was 0.08 mg/kg and this dose was found again to be very effective in clearing the trypanosomes. A reduction to 0.04 mg/kg was found to be a more viable starting dose. It was necessary to passage the original line 18 times with 16 exposures, up to a final exposure at 0.52 mg/kg to produce a resistant line of T. brucei as judged from the routine drug sensitivity test used in this work. A gross resistance factor of 13 $\left[\frac{0.52}{0.04}\right]$ was eventually obtained from the doses used. The formal pedigree of the final resistant line produced is shown in Figure 3.1. The nomenclature used in the pedigree for the resistant character was that suggested by the WHO (1978). For the notation of "exposure" a black heavy arrow is used, perpendicular to the arrows showing non-cyclical passage. The amount of drug used in mg/kg of animal is shown, and the number of days after injection at which the treatment was carried out. The stabilates prepared throughout the production of the resistant line have the following notation: the letters BEU (Biochemistry Edinburgh University), name of drug to which the stabilate has been exposed and then the drug dosage (mg/kg). It can be seen from the pedigree, that lines could be developed from the different stabilates produced.

3.1.2 The routine drug sensitivity test during the production of the resistant line

As reported earlier in this thesis L.S. forms of African trypanosomes have an absolute dependance on exogenous carbohydrate and aerobic glycolysis, linked to the reduction of molecular oxygen for the production of ATP (Ryley, 1956). Therefore it should be possible to show a correlation between the rate of oxygen consumption and the action of arsenicals, in our case melaminyl arsenicals. Moreover this correlation could be used to differentiate sensitive from non-sensitive cells. As described in Materials and Methods a routine drug sensitivity test

Figure 3.2: The inhibition of oxygen uptake in the different trypanoso populations obtained throughout the production of the resistant line of T. brucei.




3 ml of trypanosome suspension (about 0.8 mg trypanosome protein/ml) were incubated in a preequilibrated oxygen electrode with different concentrations of melarsen oxide at 37°C. The linear traces were calculated immediately after the addition of the drug and the results expressed as the percentage inhibition of oxygen uptake in untreated trypanosomes as a function of the drug concentration. Measurement was carried out in duplicated palindromic series. For details of the drug sensitivity test see Materials and Methods (Section 2.13). ●-● parent line; ◊-◊ population exposed to 0.06; □-□ 0.12; O-O 0.16; ◖-◖ 0.18; ◆-◆ 0.32; ■-■ 0.52 mg melarsen oxide/kg.

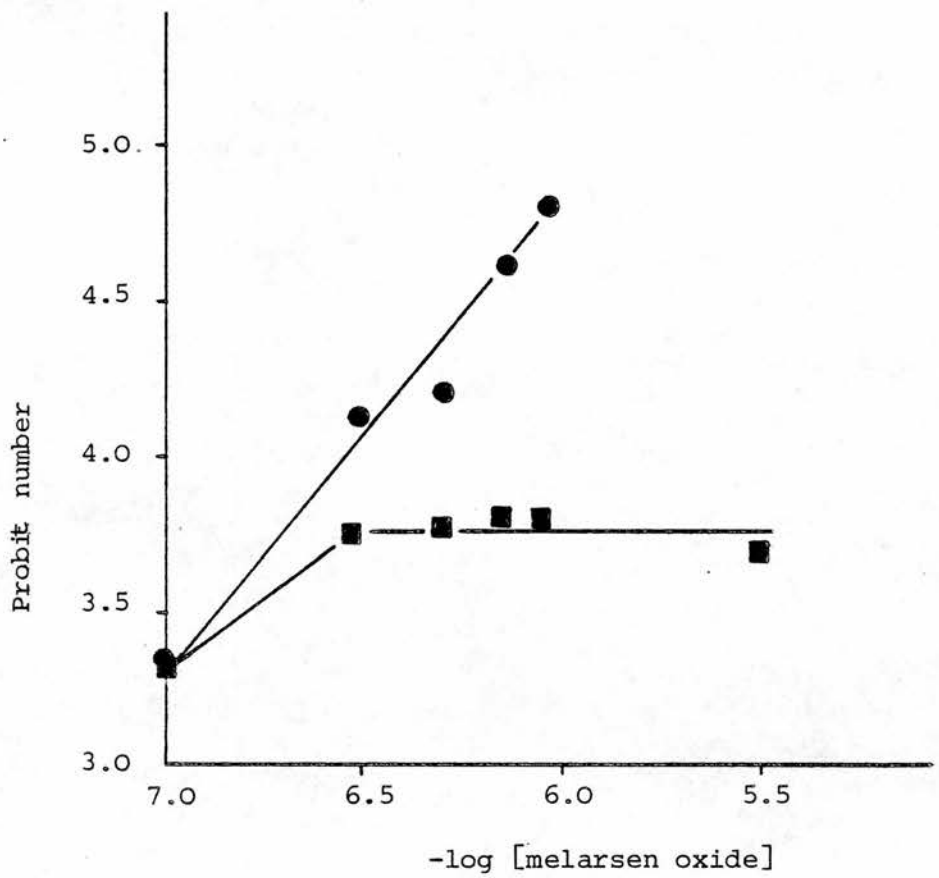
was carried out using an oxygen electrode in the presence of melarsen oxide. One slight disadvantage of the system was the non-linearity of the trace of oxygen consumption at high concentrations of the drug, when working with the parent line.

The results of these tests are shown in Figure 3.2. There is a clear difference in sensitivities between the parent line, with 40% inhibition at $9 \times 10^{-7}$M melarsen oxide and the line resistant to 0.52 mg/kg melarsen oxide with 10% inhibition of the rate of oxygen uptake at the same drug concentration. The differences between the parent line and the populations exposed to 0.06; 0.12; 0.16; 0.18 and 0.32 mg/kg melarsen oxide are not as significant as that between the parent line and the population exposed to 0.52 mg/kg melarsen oxide. As mentioned earlier and observed also in the Figure 3.2 no record is presented of inhibition above 40% for the parent line; the reason being the non-linearity of the traces of oxygen uptake. This non-linearity, at high drug concentrations, can possibly be explained in terms of multisite inhibition produced by the active agent.

3.1.3 Inhibition of the oxygen uptake; contrasted in vitro

The analysis of the development of resistance to phenyl arsenicals (trivalent tryparsamide) has been carried out in the past using the photosensitivity technique of Walker (1966). This method took advantage of the cross resistance between acriflavine and aromatic arsenicals, first reported by Jansco (1931). In the present work an attempt was made to study the development of resistance to melarsen oxide using the oxygen consumption as experimental parameter . When trypanosomes were observed using phase contrast microscopy at different intervals after the addition of the drug (under the conditions of the drug sensitivity test used), it was found that there was a decrease in the motility which appeared subjectively to be related to the decrease in the oxygen

Figure 3.3: The inhibition of the oxygen uptake in parent and resistant lines contrasted <u>in vitro</u>.




The pattern of inhibition of the parent population, ●-● and the population exposed to melarsen oxide (0.52 mg/kg) ■-■, obtained using the drug sensitivity test as in Figure 3.2 were analysed with the probit transformation as described in Materials and Methods (Section 2.24). The results are presented as the probit number as a function of the logarithm of the drug concentration.

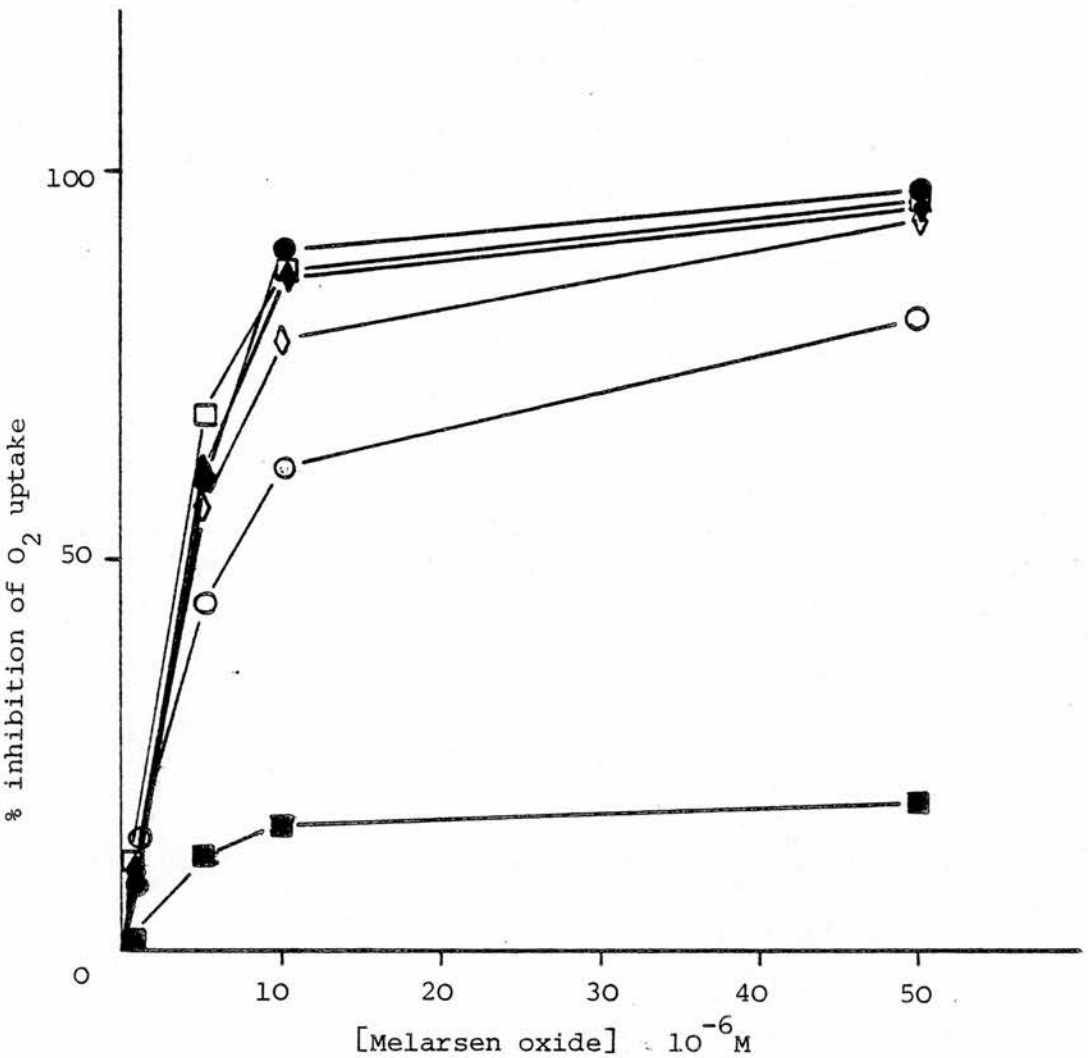
consumption. Hence it was assumed that the total oxygen uptake was related to the number of motile organism in suspension, when treated with increasing concentrations of drug and when compared to untreated sensitive trypanosomes.

Data obtained in a similar way as for the routine drug sensitivity test,are transformed for this analysis. The results are presented as the percentage of inhibition of the oxygen uptake, converted into a probit number and plotted against the negative logarithm of the concentration of melarsen oxide as in Hawking and Walker (1966). Plots of this type should produce straight lines as the probit transformation is a linearisation of drug response experiments (see Material and Methods for detail). The drug responses of parent line and the line exposed to 0.52 mg/kg melarsen oxide, are presented in this manner in Figure 3.3. The response of the parent line is linear up to the highest concentratiom of melarsen oxide used. However there is an apparently biphasic response of the resistant line. A higher slope is found at low concentrations of the drug and a plateau at higher concentrations of melarsen oxide. The probit value at which the plateau was present was approximately 3.75. As suggested by Hawking and Walker (1966) heterogeneous populations of trypanosomes with markedly different sensitivities to active agents showed complex probit plots which departed from the typical linear pattern shown in this case by the parent line of T. brucei.

3.1.4 The development of the resistant character; $O_2$ uptake inhibition in the different stabilates produced

The results of the inhibition of $O_2$ uptake in different populations of trypanosomes throughout the production of an apparently resistant line of T. brucei were presented in the last section. Stabilates of these populations were prepared for further analysis using the Warburg manometer. In all cases, trypanosomes from the first relapse after

Figure 3.4: The inhibition of the oxygen uptake in organisms from the different stabilates produced during the development of the resistant line of T. brucei.



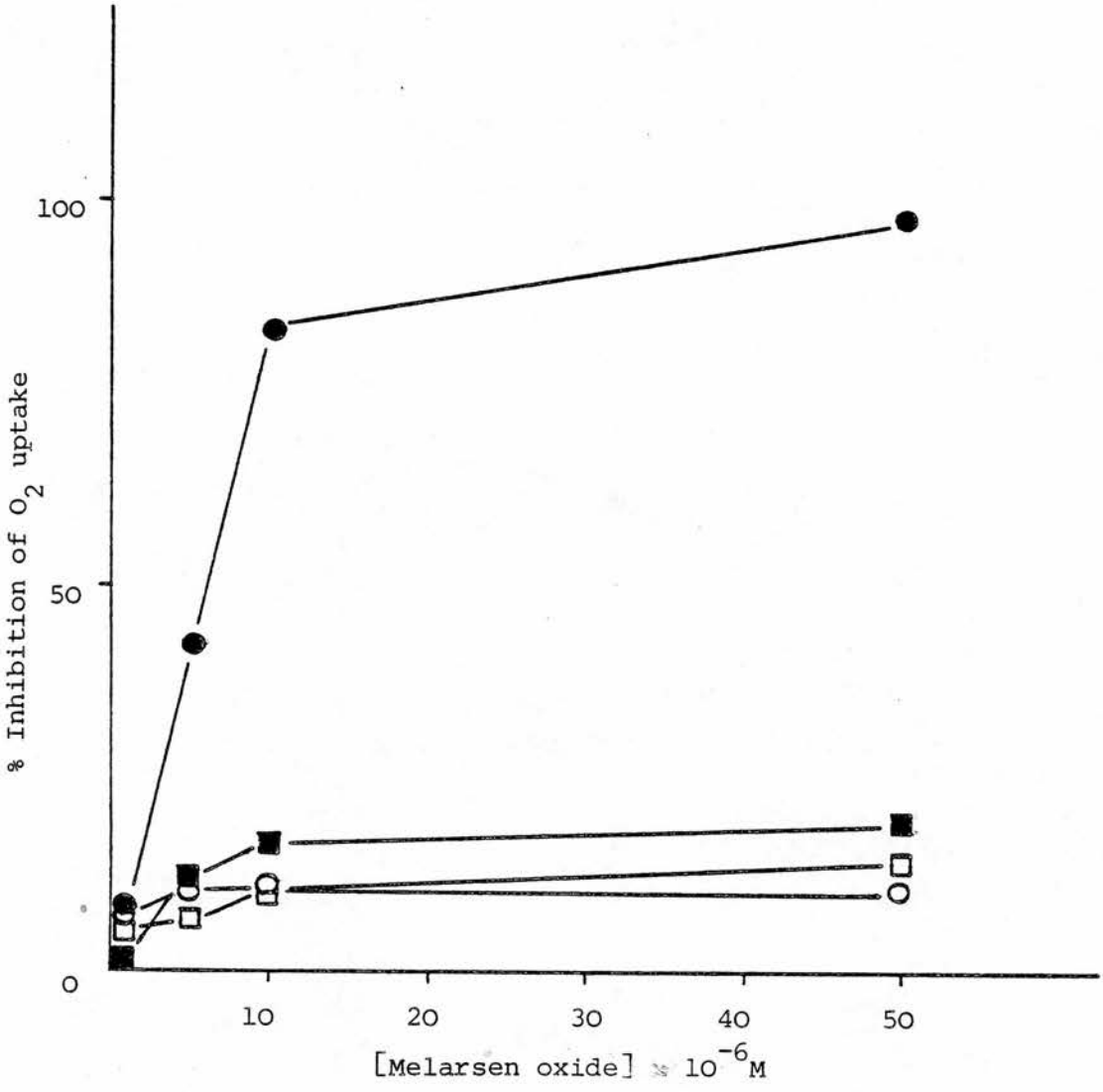

2.7 ml of trypanosome suspension (about 0.8 mg trypanosome protein/ml) were incubated in a Warburg manometer, at 25°C, incubation time 30 min, with air as the gas phase. The rest of the standard manometric conditions are described under Materials and Methods (Section 2.11). The results are from duplicate flasks and are presented as the percentage inhibition compared to untreated trypanosomes as a function of the drug concentration. ●-● parent line; ♦-♦ stabilate [BEU melarsen oxide 0.06] ; □-□ [BEU melarsen oxide 0.12]; ◊-◊ [BEU melarsen oxide 0.18]; o-o [BEU melarsen oxide 0.32]; ■-■ [BEU melarsen oxide 0.52].

injection were used for the experiments. This measure was taken to avoid any possible reversion of the resistant character to the original parent line. Oxygen uptake measured by electrode proved to be a valuable tool for routine use because of its rapid response even at low concentrations of cells. However, constant volume manometry is a more accurate and sensitive technique for the measurement of gas evolution (uptake/ production) in biological systems (Umbreit et al., 1957). Moreover, this technique was routinely used for metabolic investigation and it was considered important to compare the results obtained with those from the oxygen electrode in order to corroborate them and also to have a coherent body of data obtained with a single technique.

The results of the inhibition of oxygen uptake by melarsen oxide in some of the different stabilates prepared are shown in Figure 3.4. The patterns of inhibition were obtained using the standard manometric techniques described in Material and Methods.

Again it is found that the inhibition patterns of the lines exposed to relatively low concentrations of melarsen oxide ( stabilates[BEU melarsen oxide 0.06; 0.12; 0.18]) are similar to the inhibition of the parent line, with the $I_{50}$ for inhibition of oxygen uptake lying between 4 and 5 x $10^{-6}$M melarsen oxide. With regard to the meaning of the $I_{50}$ values when using Warburg mamometry and the differences found with respect to the $I_{50}$ values obtained in the routine drug sensitivity test, a comment is made in the Discussion,in the section on the development of the resistant character. A small but apparently significant difference is found for the stabilate [BEU melarsen oxide 0.32] with an $I_{50}$ of 7 x $10^{-6}$M melarsen oxide and a maximal inhibition of 80% at high concentrations of the drug. The pattern changed completely for the relapse from the stabilate [BEU melarsen oxide 0.52], with a maximal inhibition of 20%. The appearance of the resistant character

Figure 3.5: The inhibition of the oxygen uptake in different populatio of stabilate [BEU melarsen oxide 0.52] after serial passage in rats.




A suspension of trypanosomes( about 0.8 mg protein/ml) were incubated using the standard manometric conditions. These conditions and the method of trypanosome isolation are as described in Materials and Methods (Section 2.5). The organisms used are isolated after: ■-■ 1st passage; O-O 4th passage; □-□ 13th passage and compared with the parent line ●-●.

is therefore apparently discontinuous during the production of the resistant line as judged by the inhibition patterns of the different stabilates.

From now on the relapses obtained from stabilates of the population exposed to 0.52 mg/kg melarsen oxide are called the Resistant Line and most of the comparative work has been done using the latter and the original parent line. For the purposes of this work, the stabilates [BEU melarsen oxide 0.06; 0.12; 0.16] are considered to have "low resistance", stabilates [BEU melarsen oxide 0.18; 0.32] "intermediate resistance" and the stabilate [BEU melarsen oxide 0.52] "high resistance". No attempt was made to increase further the level of resistance.

3.1.5 The stability of the resistant character in vitro

Drug resistance, once fully developed, is normally a relatively stable character even in the absence of drug pressure (Murgatroyd and Yorke, 1937). However, stability may vary from one line to another (Bishop, 1959). One example of the stability of resistance to organic arsenicals is reported by Hawking (1963) who described a line of T. rhodesiense resistant to atoxyl which remained resistant for 30 years, after serial passage.

The next stage in this work was to check the stability of the resistant line during serial passage, in the absence of drug pressure. The sensitivity to melarsen oxide was estimated using the standard manometric system after the 4th and the 13th passages. The results are shown in Figure 3.5. It may be seen that the resistant line developed after the maximal dose of melarsen oxide used (0.52 mg/kg) was stable up to the 13th passage in rats as judged by the inhibition patterns. No attempt was made to study the degree of stability of the lines with "low and intermediate" resistance. However, it has been suggested that

Table 3.1: The action of melarsen oxide on the motility of T. brucei in vitro

Parent line

| Melarsen oxide x $10^{-6}$M | Time (minutes) 10 | 20 | 30 |
|---|---|---|---|
| 0 | +++ | +++ | +++ |
| 1 | +++ | ++ | ++ |
| 5 | ++ | ++ | + |
| 10 | ++ | +o | +o |
| 50 | + | +o | +o |

Resistant line

| Melarsen oxide x $10^{-6}$M | 10 | 20 | 30 |
|---|---|---|---|
| 0 | +++ | +++ | +++ |
| 30 | +++ | +++ | +++ |
| 80 | +++ | +++ | +++ few dead |
| 150 | +++ | +++ | +++ few dead |

LS trypomastigotes were incubated in PSGA buffer (about 0.8 mg trypanoso protein/ml buffer) at 25°C. The motility was recorded as a function o time by observation under phase contrast microscopy. Details of the motility test is as described under Materials and Methods (Section 2.7)

lines with relatively low levels of resistance to arsenicals are relatively unstable (Schueler et al., 1947; Hawking and Walker, 1966).

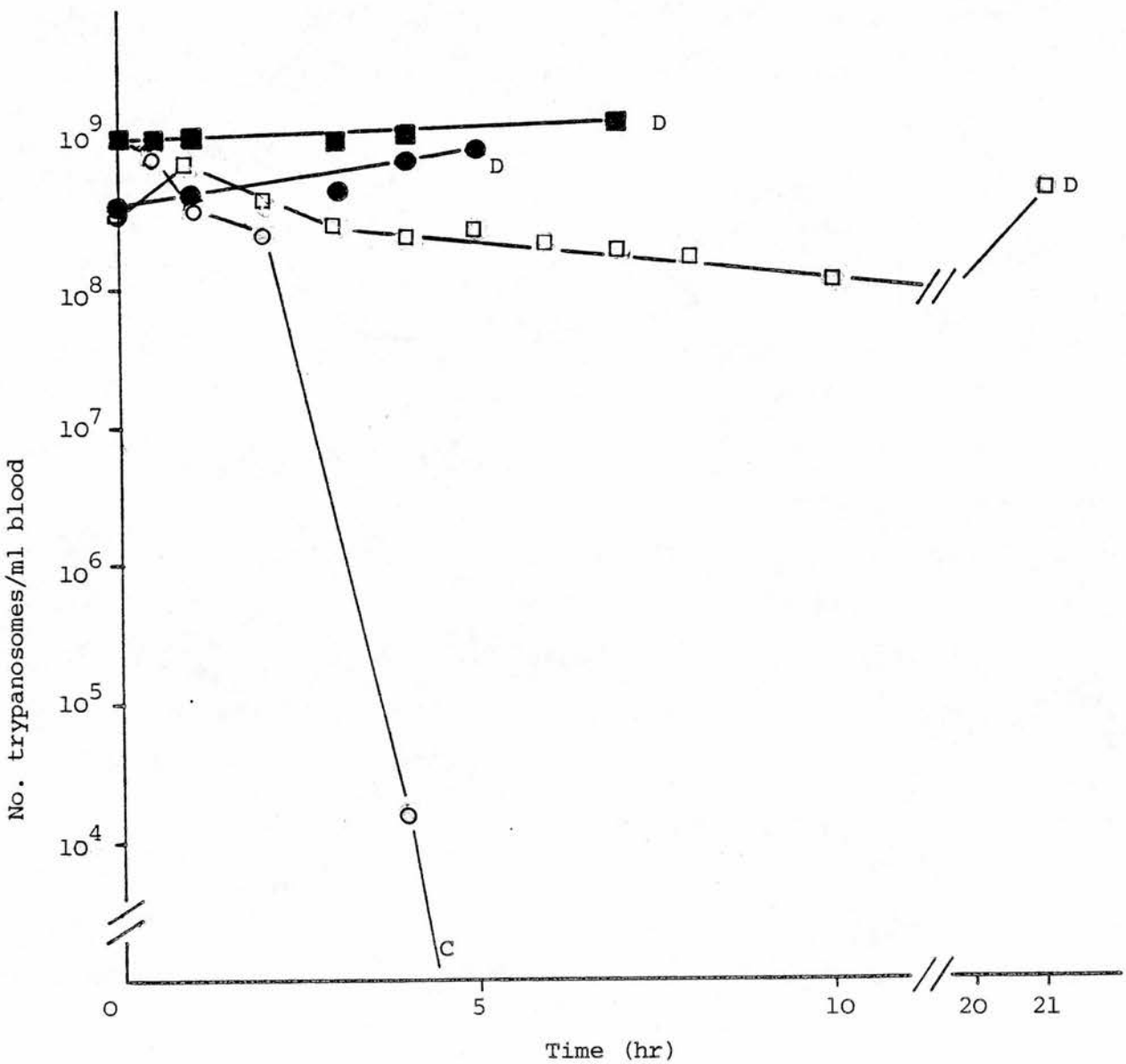
3.1.6 The effect of melarsen oxide on motility in vitro

It has already been reported that the reduction in oxygen uptake is accompanied by a reduction in motility of the trypanosomes, the reason being that trivalent arsenicals are rapidly cytotoxic due to the focal action on aerobic glycolysis. The estimation of motility, although subjective, gives valuable qualitative information about the sensitivity of the parasites to the active agent.

By looking at Table 3.1, it can be seen that melarsen oxide has the expected effect on the motility of the parent line. However, after 30 minutes of incubation at the maximum concentration of drug used ($50.10^{-6}$M) a few organisms retained some movement. The situation is completely different with the resistant line which did not respond to the action of the drug. At high concentrations of the drug and towards the end of the experiment a few organisms were observed apparently dead as judged by the lack of motility. (Note that the concentration of melarsen oxide and the concentration of trypanosomes were the same as for the manometric experiments.)

With regard to the small percentage of organisms which either did not die or did die in the parent and resistant line respectively, it has been suggested (Hawking and Walker, 1966) that a population of trypanosomes has a "normal" distribution of sensitivities to the chemotherapeutic agents with which the population is treated. This is apparently shown in the motility test presented as there were a few organisms in both lines of trypanosome which did not respond as the rest of the population to the active agent.

Figure 3.6: The action of melarsen oxide <u>in vivo</u> on trypanosomes in parent and resistant lines.




5 µl of blood from the tail of infected rats with terminal parasitaemia of the parent and the resistant lines was collected into 3 ml of PSG buffer. Treated rats were injected intravenously (using the penile vein) with melarsen oxide (0.52 mg/kg) in saline (0.3-0.5 ml). The number of motile organisms was calculated in triplicate using haemocytom and presented as a function of time. ●-● parent line; ■-■ resistant line; O-O parent line + melarsen oxide; □-□ resistant line + melarser oxide; D stands for death of the animal; C for cured animal.

3.1.7 The effect of melarsen oxide on motility in vivo

All the metabolic experiments described so far, were carried out in vitro. The next objective was to evaluate the resistant character in vivo, in laboratory animals. Rats with terminal parasitaemia of either parent or resistant line were treated intravenously with the highest concentration of melarsen oxide used during the production of the resistant line (0.52 mg/kg), and the number of motile trypanosomes present in blood samples were estimated by haemocytometry for several hours. The results are shown in Figure 3.6. It may be seen that both controls of untreated rats with parent or resistant line infections died within 5 to 7 hours after the experiment was started. Conversely the parent line is cleared almost completely from the bloodstream in approximately five hours. The resistant line showed a small reduction in the rate of growth and the death of the animals occurred the following day.

3.1.8 Summary

For the purpose of this work on the rationale behind resistance to arsenicals, a line of T. brucei resistant to one of these drugs was needed (melarsen oxide). Such a resistant line was prepared using the short passage method. The new resistant line as reported in the formal pedigree was obtained after 18 passages (10 weeks) with 16 exposures to melarsen oxide. The maximal dose used was 0.52 mg/kg of animal. The line had a gross resistance factor of 13 fold when compared with the parent line.

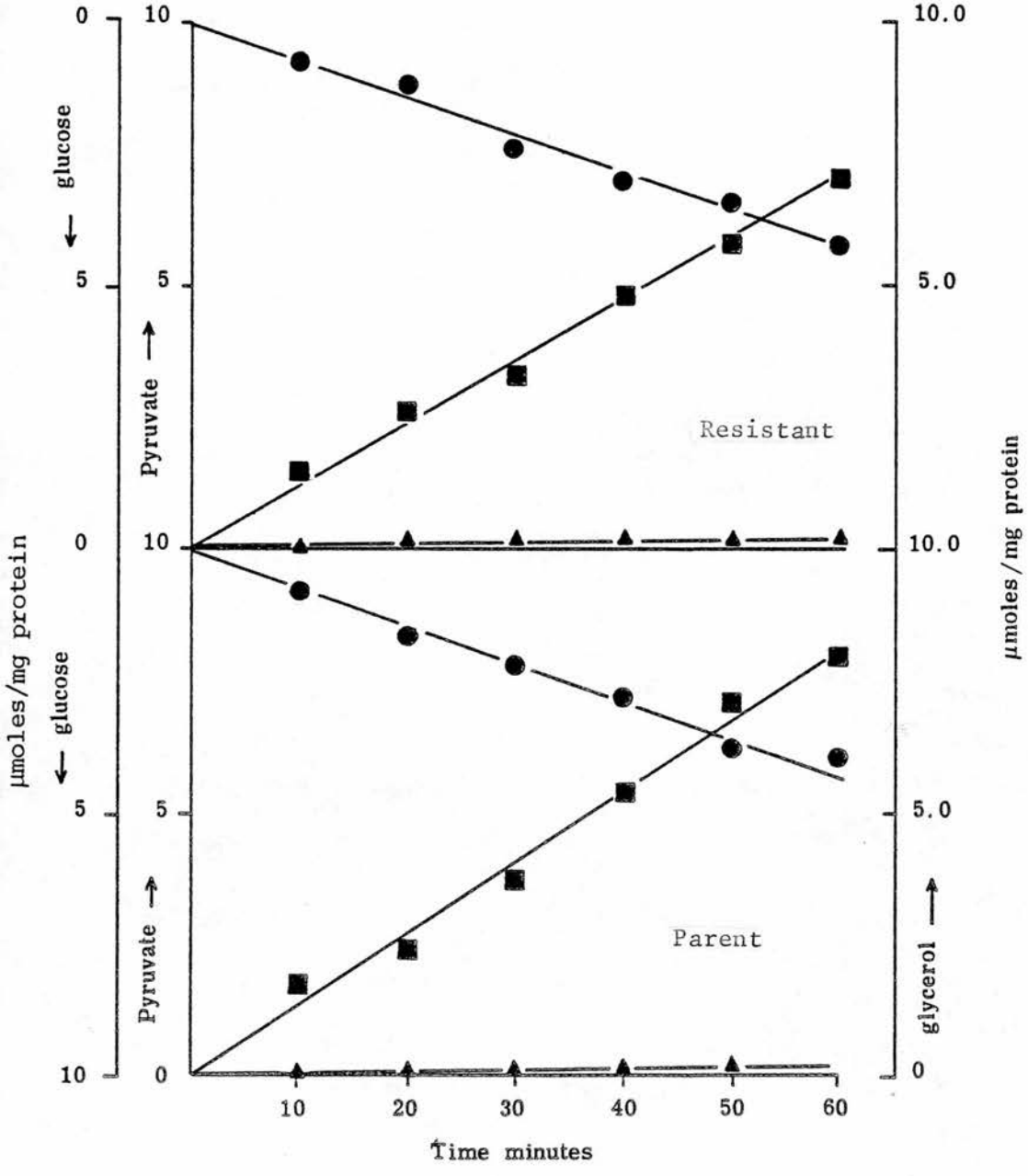
As it was a newly developed resistant line, it was necessary to check certain general characteristics e.g. the stability and homogeneity of the resistant character and the behaviour of the resistant line in vivo and in vitro under pressure of the drug.

With the help of a routine drug sensitivity test and further studies with constant volume manometry, it was found that the new line was apparently developed in stages. It was also found that the line may be a mixed population of trypanosomes with a decreased sensitivity to melarsen oxide. The resistant character was stable after 13 serial passages.

Finally it was found that the resistant line proved to be effective in killing rats under drug pressure when compared to the parent line which did not kill the host.

The following chapters of this thesis are dedicated to the study of the resistant character developed in this new line of T. brucei.

Figure 3.7: Preliminary experiment on the comparative carbohydrate metabolism of parent and resistant lines in vitro.




14 ml of trypanosome suspensions with about 13 mg of trypanosome protei were incubated in 25 ml conical flasks plugged by a rubber stopper. The buffer conditions were PSGA with 15 mM glucose and 150 mg% BSA (Brohn and Clarkson, 1978). Prior to initiation of the experiment, the flasks were gased with $O_2/CO_2$ (95%/5%) through a vent in the rubber stopper. The gas mixture was humidified by bubbling through water prio to passage to the flasks. The systems were incubated at 25 °C in a water bat with shaker; the experiment was terminated by removing aliquots (1 ml) from the flasks at 10 minute intervals and centrifuging these immediate in a Beckman microfuge for 1 min. The metabolite estimation was perfor as described in Materials and Methods (Section 2.12). The results obtained are shown as a function of time: ●-●, glucose; ▲-▲, pyruvate; ■-■, glycerol.

## 3.2 Metabolic studies

As has been described in the Introduction,a metabolic change has been suggested on several occasions as a possible mechanism to explain the development of resistance to arsenicals. Although this idea is not shared by all authors, it remains as a feasible possibility in Trypanosomes fast to these drugs. The suggestion of a metabolic bypass was one of the earliest explanations for the phenomenon in arsenical fast trypanosomes, as these drugs are accepted metabolic inhibitors. The main objective of this chapter was to investigate the possibility of a metabolic change as a result of the development of the resistant character. The basic glycolytic parameters were evaluated in the parent and in the new drug fast line of T. brucei.

### 3.2.1 Preliminary experiments on comparative metabolism

Trypanosomes of the brucei group, which depend exclusively on glycolysis for generation of ATP, produce pyruvate from glucose under aerobic conditions in quantitative amounts (Ryley, 1956; 1962; Grant and Fulton, 1957). Recently the end products of aerobic metabolism of LS forms in the parent line of T. brucei used were studied and compared with the general metabolism of other representatives of this group. No significant differences were found (Hammond, 1979).

The results presented below are measurements of glucose consumption, pyruvate production and glycerol production. These were obtained in order to compare parent and resistant line. A suspension of trypanosomes in PSGA buffer, (for detail see Material and Methods), from both lines of T. brucei were incubated in small conical flasks, in such a way that a high surface area-volume ratio was obtained. The system was incubated under agitation at controlled temperature with $O_2/CO_2$ as the gas phase. At the required time, flasks were deproteinised and the estimation of the metabolites described above was carried out. For the method of estimation of metabolism see Material and Methods. Figure 3.7 shows the

Table 3.2: Preliminary data on the carbohydrate metabolism and carbon balance of parent and resistant lines.

| | Metabolite changes (nmol $min^{-1}$ mg $protein^{-1}$) | |
|---|---|---|
| | Parent | Resistant |
| glucose consumption | $69.79 \pm 3.20$ | $70.83 \pm 4.15$ |
| pyruvate production | $137.50 \pm 5.40$ | $116.67 \pm 4.82$ |
| glycerol production | less than 4.0 | less than 4.0 |
| | **Metabolic ratios** | |
| pyruvate/glucose | 1.97 | 1.65 |
| glycerol/glucose | 0.06 | 0.06 |
| | **carbon recovery** | |
| $\left(\frac{2 \text{ Pyruvate production}}{\text{glucose consumption}}\right)$ X 100 | 98.51 | 82.36 |

The slopes of the rates of metabolite production/consumption presented in Figure 3.7 were calculated as described in Section 2.24. From the results obtained the different metabolic ratios and carbon recoveries were estimated; mean $\pm$ standard deviations of a typical experiment are shown for the metabolite changes.

data for metabolite changes under the conditions described. Isolated trypanosomes, in a simple buffer system at 25°C took up glucose and produced pyruvate in a linear fashion for up to 60 minutes.

The slopes of the regression lines from these data (i.e. ratios of utilisation/production) are compared in Table 3.2. The rates of glucose uptake in both lines were the same, although the rate of pyruvate production in the resistant line was apparently lower, when compared with the parent line.

The production of trace amounts of glycerol was also observed. This is considered to be of minor importance in the experiment as it did not increase with time. The aerobicity of the system during incubation is therefore ensured, as trypanosomes under anaerobic conditions produce equimolar amounts of pyruvate and glycerol (Ryley, 1962; Hammond, 1979). The molar ratios and the carbon recovery for the parent line showed that pyruvate accounted quantitatively for the glucose used. On the other hand the molar ratios and the carbon recovery suggest a smaller yield of pyruvate from glucose in the resistant line. It should be pointed out that the level of glycerol was again negligible, and therefore the lower yield was not due to anaerobic metabolism by the parasites. With regard to the presence of glycerol, the possibility of transient anaerobic conditions during the handling of the trypanosomal suspensions is suggested as the possible reason for the presence of this metabolite.

Finally as a result of the small but detectable difference found in the carbon balances of the two lines, as shown by the difference in the pyruvate/glucose ratios, a more comprehensive approach was taken in order to study in more detail the carbohydrate metabolism of parent and resistant lines of T. brucei.

Table 3.3: The general carbohydrate metabolisms in T. brucei parent and resistant lines using the standard manometric conditio

| | Parent line | Resistant line |
|---|---|---|
| $O_2$ consumption | $65.63 \pm 0.72$ (3) | $70.51 \pm 2.12$ (3) |
| glucose consumption | $69.61 \pm 1.56$ (4) | $73.13 \pm 0.75$ (4) |
| pyruvate production | $136.89 \pm 2.06$ (4) | $126.44 \pm 1.78$ (4) |
| glycerol production | $5.97 \pm 0.87$ (2) | $4.13 \pm 1.27$ (2) |
| $CO_2$ production | Nil (3) | $7.16 \pm 0.5$ (3) |

(Rates of metabolite production or consumption in nmol $min^{-1}$ mg protei

2.7 ml of trypanosome suspensions (about 0.8 mg trypanosome protein/ml) in PS buffer with glucose ($4.0 \times 10^{-3}$ M) and BSA (0.5 mg/ml) were incubated using the standard manometric conditions described in Section 2.11, at 25°C. The linear rates of gas evolution were measure over a period of 30 minutes. After deproteinisation the metabolites involves were determined as in Section 2.12. The figures are presente as the mean $\pm$ the standard error of the mean; in parenthesis the number of analysis.

### 3.2.2 General carbon balance in parent and resistant lines

As discussed earlier in this chapter, the metabolism of lines of trypanosomes resistant to arsenicals has resulted in a rather inconclusive picture. On one side some workers advocate no apparent change in general metabolism (Reiner et al., 1936; Fulton and Stevens, 1945; Harvey, 1949). On the other hand, minor modifications in the general catabolic routes of resistant parasites, have been shown (von Brand et al., 1953; Williamson, 1953a). As suggested in the last section a more comprehensive approach should be taken in order to assign some significance to the differences found in the general carbon balance of parent and resistant parasites. Hence the Warburg Manometric system was used for further investigation.

The results of these experiments are shown in Table 3.3. Note that oxygen consumption has been included in the data. The figures obtained for pyruvate production and glucose utilisation were similar to those obtained in the preliminary experiment. No significant differences were observed when comparing glucose or oxygen utilisation rates of parent and resistant line.

With regard to pyruvate production, an apparently lower rate was again observed in the resistant line, but the standard deviations observed precluded the assigning of significance to those results at this stage. The significance of these results is discussed in detail in the next two sections when the molar ratios and carbon recoveries are evaluated.

Monomorphic, laboratory adapted lines of T. brucei should not produce $CO_2$, as a final product of their metabolism (Flynn and Bowman, 1973). However the possibility of $CO_2$ evolution was evaluated in both lines for comparative purposes. The Direct Method of Warburg was used for the estimation of $CO_2$; details of the method are found under

Table 3.4: The molar ratios and carbon recoveries from glucose in the aerobic metabolism of T. brucei in parent and resistant line organisms.

a) Molar ratios

| | Parent line | Resistant line |
|---|---|---|
| oxygen/glucose | $0.98 \pm 0.04$ (3) | $0.94 \pm 0.03$ (3) |
| pyruvate/glucose | $1.97 \pm 0.02$ (4) | $1.73 \pm 0.02$ (4) |
| pyruvate/oxygen | $2.08 \pm 0.05$ (3) | $1.84 \pm 0.08$ (3) |
| glycerol/glucose | $0.09 \pm 0.01$ (2) | $0.05 \pm 0.01$ (2) |

b) Carbon recoveries %

| | Parent line | Resistant line |
|---|---|---|
| glucose | 100 | 100 |
| pyruvate | $98.33 \pm 0.81$ (4) | $85.45 \pm 1.19$ (4) |
| glycerol | $4.29 \pm 0.67$ (2) | $2.28 \pm 0.44$ (2) |
| $CO_2$ | Nil (2) | $9.79 \pm 0.97$ (3) |
| Mean total recovery % | 102.62 | 97.52 |

The data presented in Table 3.3 expressed as molar ratios and carbon recoveries from glucose in the different experiments performed. Results presented as the mean $\pm$ standard error of the mean; number of determinations in parenthesis.

Material and Methods. It was observed that the PSGA buffer used during these experiments, even when freshly prepared binds some atmospheric $CO_2$, possibly following the equation:

$$Na_2HPO_4 + CO_2 + H_2O \rightleftharpoons NaH_2PO_4 + NaHCO_3$$

This error was controlled by subjecting the thermobarometers to exactly the same conditions as the experimental barometers and correcting for the presence of $CO_2$ in the buffer after acidification of the contents of the experimental flasks. The results of these experiments are shown in Table 3.3. A typical response for the parent line was found, without measurable evolution of $CO_2$. However, the resistant line showed a measurable production of this metabolite, with a respiratory quotient (RQ) of approximately 0.10. Therefore, although the difference in the rates of pyruvate production were small in parent and resistant lines, the formation of a second product from the metabolism of glucose by the resistant line seems to be certain.

3.2.3 Molar ratios and carbon recoveries from parent and resistant lines

To illustrate the distribution and recoveries of end products in both parent and resistant line, the molar ratios and recovery of each metabolite were calculated, the objective being to stress any possible differences between the two lines of trypanosomes.

From the data shown in Table 3.4a,b, it may be suggested that the aerobic metabolism of the parent line of T. brucei is similar to the other bloodstream forms of the subgenus Trypanozoon (Flynn and Bowman, 1973; Brohn and Clarkson, 1978). There was a quantitative formation of almost two moles of pyruvate per mole of glucose consumed, with an overall yield of around 98%. The overall scheme of metabolism is therefore consistent with the one presented in Figure 1.2. However, when looking at the results in Table 3.4a, some differences may exist

Table 3.5: The levels of significance of the different molar ratios - for parent and resistant lines obtained using the "t stude test".

| Ratio | t value | Degrees of freedom | Probability | Conclusi |
|---|---|---|---|---|
| $O_2$/glucose | 0.674 | 2 | <<0.10 | ND |
| $P_y$/glucose | 3.79 | 3 | <0.02 | SD |
| $P_y$/oxygen | 4.15 | 2 | <0.05 | SD |

ND = no difference

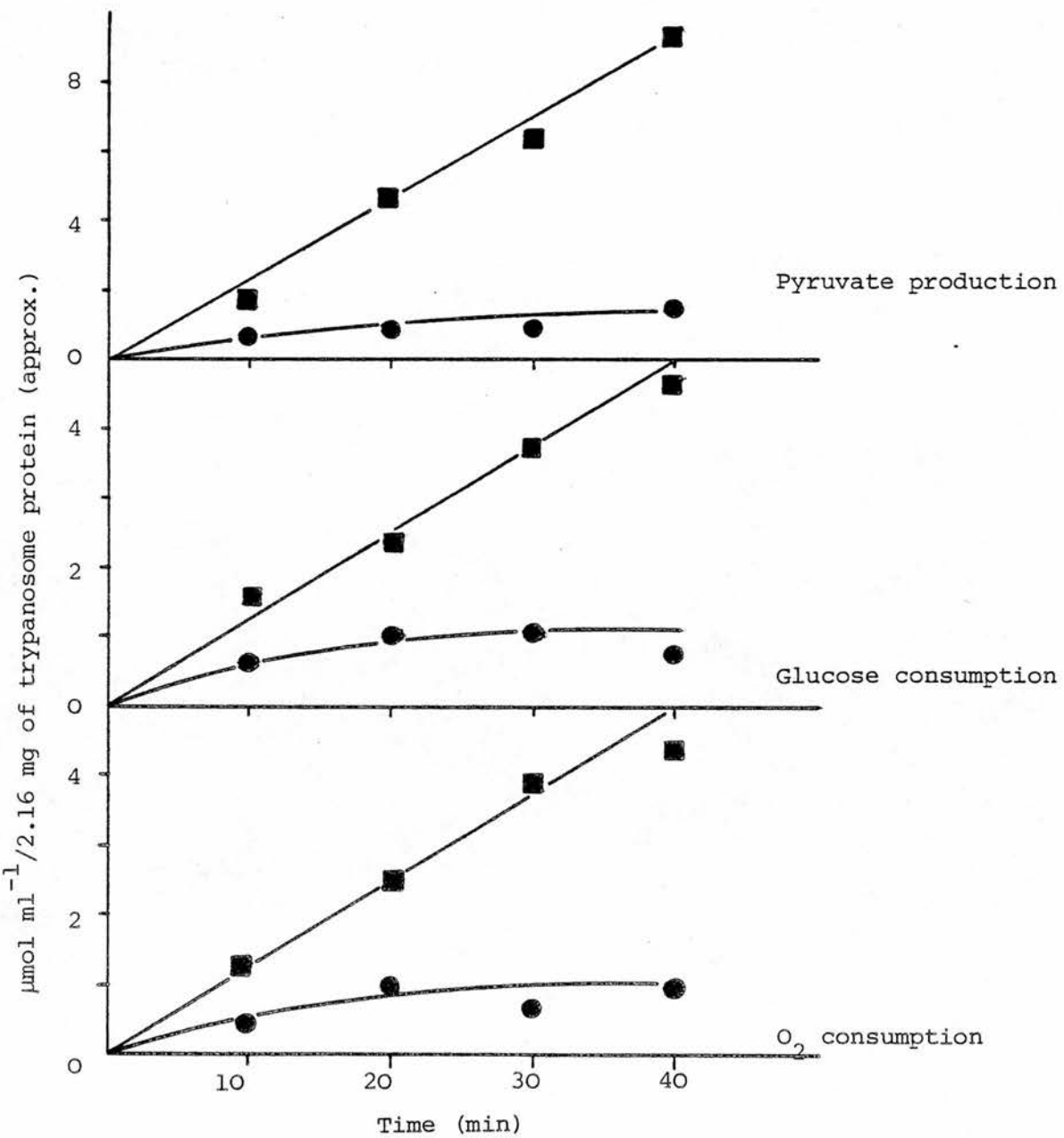
SD = significant difference

The results are presented in Table 3.4a were analysed using the "Paired student test". After calculation of the "t" value these were transformed into probability values by entry in the Tables specially prepared for this purpose, at the appropriate degrees of freedom.

with respect to the pyruvate/glucose and pyruvate/oxygen ratios in the resistant line of trypanosome. No differences were observed however in the $O_2$/glucose ratios.

It is clear, as shown in Table 3.3 that a relatively large standard deviation was found for the metabolic parameters, which may be produced by the variation in the figures obtained for the different metabolites from one metabolic experiment to another. Nevertheless, similar results were consistently obtained showing lower pyruvate yields from the resistant line. Therefore, in order to determine whether there was a difference in the carbon balance of parent and resistant line, the "student" t test was used, making a paired comparison of the data obtained in the different experiments. The results are shown in Table 3.5. The data shows that the differences found with regards to pyruvate/oxygen and pyruvate/glucose ratios are apparently significant at the calculated levels of confidence. Therefore, the resistant line may have a small modification in its general metabolism, which involves the production of lower levels of pyruvate when compared with the parent line. Different alternatives for the fate of this pyruvate are put forward in more detail under Discussion. However, the possibility of further metabolism of pyruvate with production of $CO_2$ is favoured.

Figure 3.8: The effect of the incubation time on the metabolism of carbohydrate in parent and resistant lines in the presence of melarsen oxide, using the Warburg manometer.




2.7 ml of trypanosome suspension (about 0.8 mg trypanosome protein/ml) was incubated in a Warburg manometer at 25°C, with air as a gas phase and 6 x $10^{-6}$ M melarsen oxide; the rest of the standard manometri conditions are as described under Materials and Methods (Section 2.11). Incubations were terminated every 10 minutes by addition of perchloric acid (0.3 ml, 6M) from the side-arms of the flasks. Deproteinisation and estimation of metabolites are as described under Materials and Methods; duplicated results are presented as a function of time for the different metabolites estimated. ●-● parent line; ■-■ resistant line.

## 3.3 The action of melarsen oxide on the metabolism of parent and resistant lines

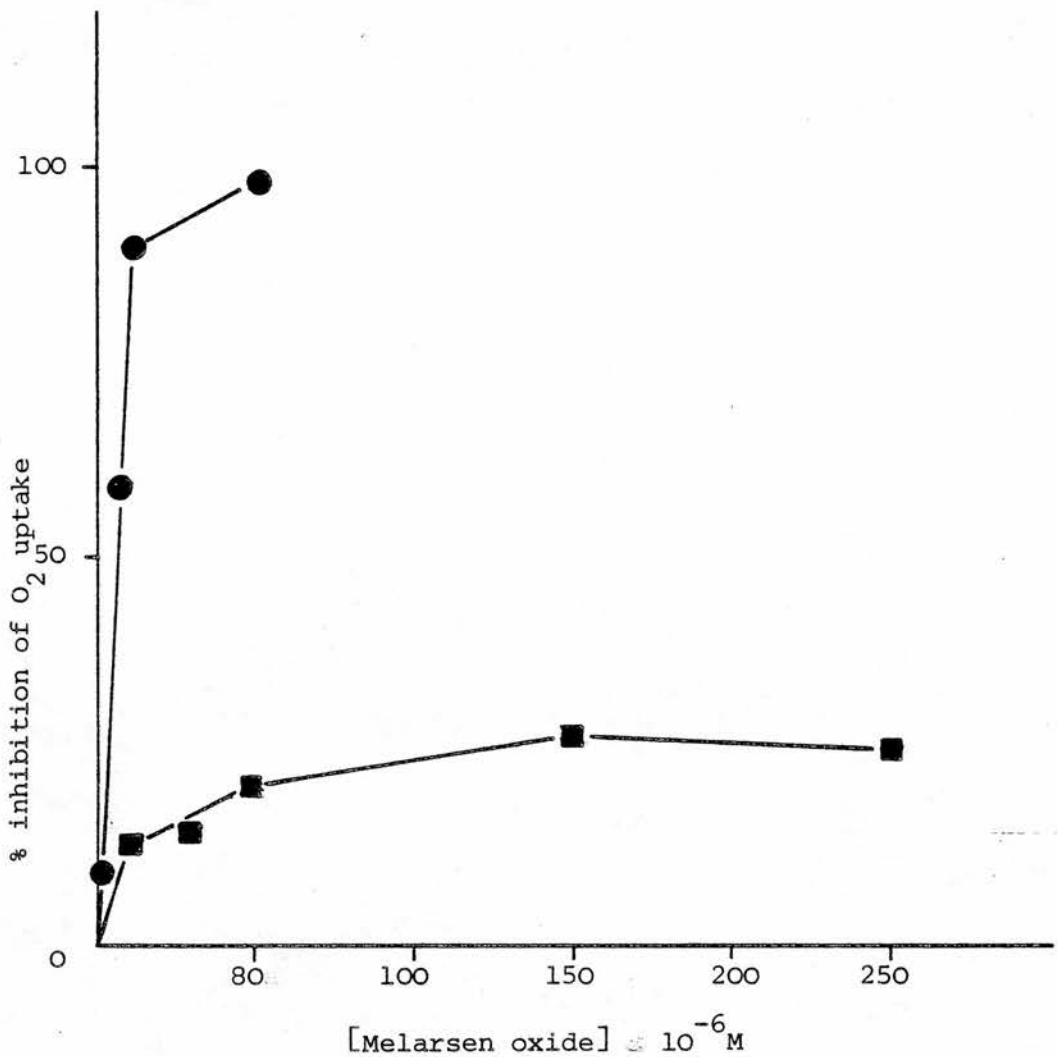
A difference in the carbohydrate metabolism of the resistant line of T. brucei has been confirmed with the metabolic experiments performed in the last sections. Nevertheless, this apparently minor metabolic change may not explain the resistance factor observed (13 times). At the same time this metabolic difference does not explain the different patterns of inhibition of $O_2$ uptake observed for parent and resistant line (see Figure 3.4). Therefore a series of experiments were performed under drug pressure to determine whether these metabolic changes may alone explain the development of the resistant character.

### 3.3.1 The effect of incubation time on the metabolism of parent and resistant lines in the presence of melarsen oxide

In the absence of melarsen oxide the metabolite production and consumption were linear under the experimental conditions used. The question arose as to whether metabolism follows the same pattern in the presence of the drug.

The standard manometric conditions were used in this experiment. The method for deproteinisation and estimation of metabolites are described in Material and Methods. The concentration of drug used for both parent and resistant line was chosen from the initial oxygen inhibition experiments and it was $5 \times 10^{-6}$ M melarsen oxide (see Figure 3.4). The results of metabolite utilisation and formation as a function of time are shown in Figure 3.8. It was found that the glucose and oxygen consumption and pyruvate production were linear in the resistant line. However in the parent line the same parameter showed a plateau after 15-20 minutes. The standard time of 30 minutes was used in the next set of experiments, to allow maximisation of any difference in metabolite ratios between the two strains.

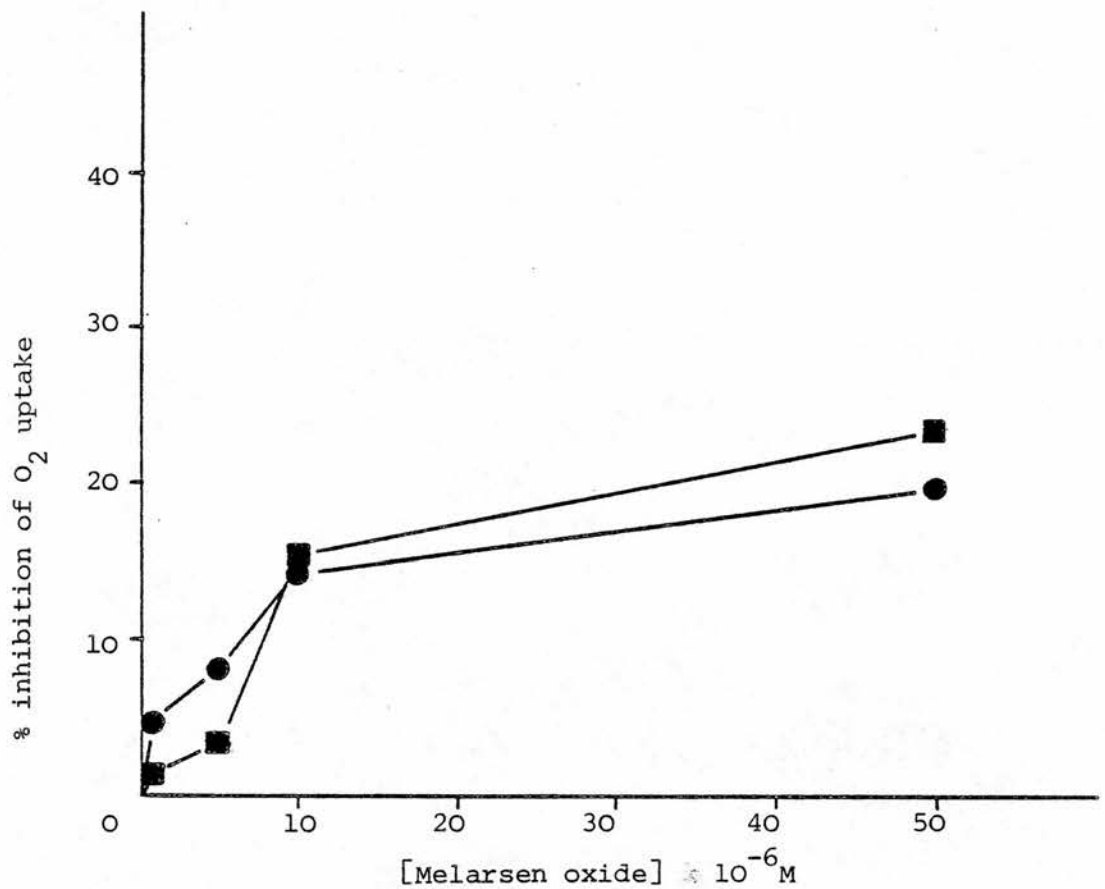
Figure 3.9: The effect of melarsen oxide on the oxygen uptake by whole cells in parent and resistant lines.




2.7 ml of trypanosome suspension (about 0.8 mg trypanosomes protein/ml) was used for this experiment. The standard manometric conditions were used at 25°C with air as the gas phase, incubation time 30 minutes.

Routines used for deproteinisation and estimation of metabolites are as described under Materials and Methods (Sections 2.11 and 2.12). Results from duplicated flasks are presented as a function of the drug concentration: ●-● parent line; ■-■ resistant line.

Figure 3.10: The effect of melarsen oxide on the oxygen uptake by water lysates in parent and resistant lines.



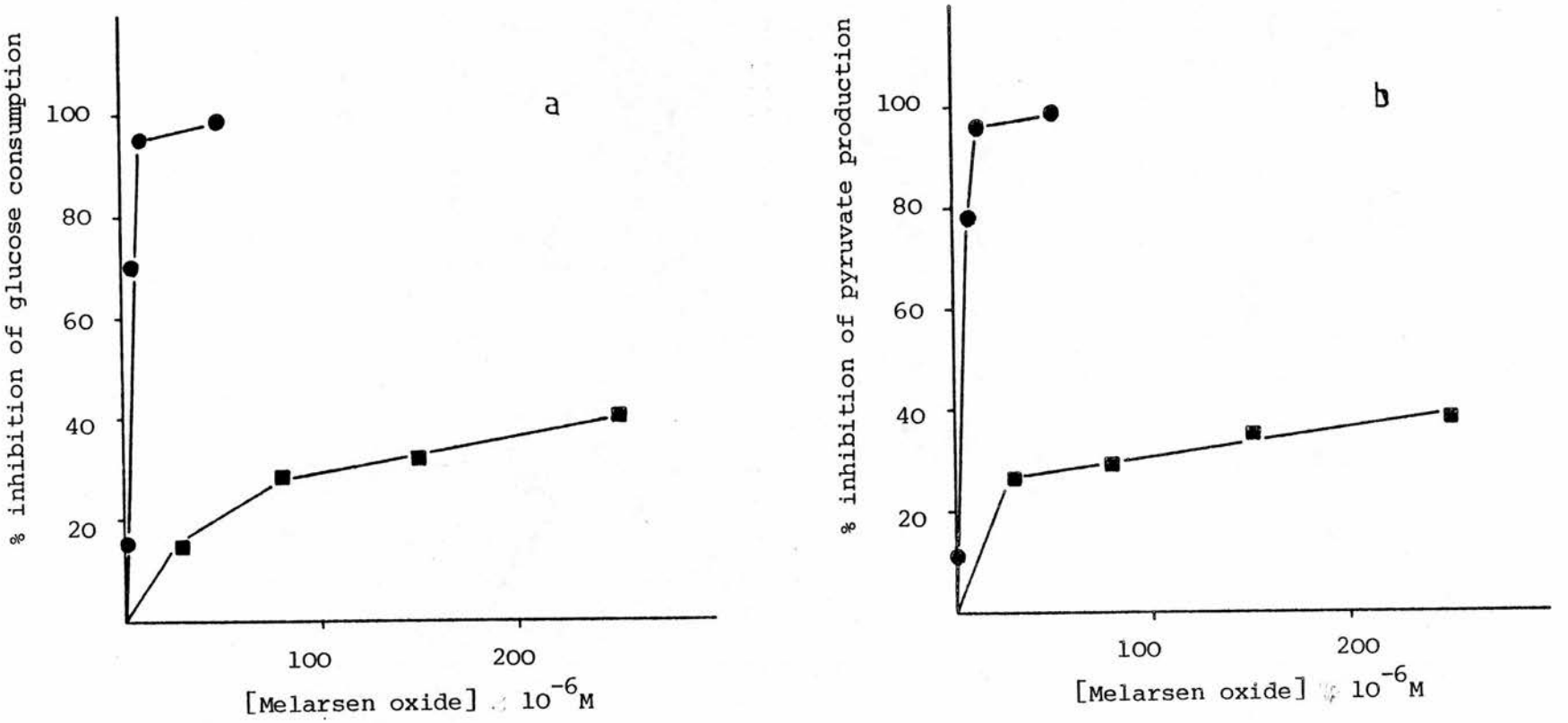

2.7 ml of water lysates in minimal fortified medium (Flynn and Bowman, 1974) were used for this experiment; details of the medium are as described under Materials and Methods (Section 2.8.2). The rest of the experimental conditions were as in Figure 3.9. Results from duplicate flasks are presented as a function of the drug concentration. ●-● parent line; ■-■ resistant line.

3.3.2 The effect of melarsen oxide on the oxygen uptake in whole cells and water lysates

As discussed in the chapter on the production of the resistant line, LS trypanosomes rely completely on aerobic glycolysis and this pathway is sensitive to the action of trivalent arsenicals. A relationship between oxygen consumption and drug concentration was therefore expected. A correlation was found and it was used to differentiate the parent and the resistant line, in terms of the lack of inhibition found with the routine drug sensitivity test (see Figure 3.2).

For investigation of whole cells the standard manometric conditions were used with a range of drug concentrations. Trypanosomes from the first relapse after inoculation with stabilates of parent and resistant lines were used. No differences in the resistant character were found under the conditions of cryopreservation used for up to 7 months storage. The results of these experiments with whole cells are shown in Figure 3.9. Total inhibition of $O_2$ uptake by the parent line is observed but incomplete inhibition is found with the resistant line. An $I_{50}$ value for $O_2$ uptake of approximately $5 \times 10^{-6}$M melarsen oxide is observed for the parent line while the maximum inhibition in the resistant line is approximately 25% at 83 times the concentration of drug to produce 50% inhibition in the parent line. The patterns of inhibition of oxygen uptake by water lysates using the minimal fortified medium of Flynn and Bowman (1974) (described in Material and Methods) and the standard manometric conditions, are presented in Figure 3.10. It may be seen that the inhibition patterns did not show a significant difference between parent and resistant line. Therefore it is suggested that some common point of inhibition may be sensitive in both parent and resistant line.

Figure 3.11: The effect of melarsen oxide on the pyruvate production and glucose consumption in parent and resistant lines.




All the experimental conditions are as described in Figure 3.9. ●-● parent line; ■-■ resistant line.

With respect to the marked difference of inhibition in the whole cells and the similarity of the pattern with water lysates, it is suggested that a problem of accessibility of the drug for its final receptor is involved. It was observed that the extracellular concentration of melarsen oxide needed to produce 50% inhibition of $O_2$ uptake by whole cells of the parent line is considerably less than the concentration required to produce even 20% inhibition in water lysates. This observation may be explained in terms of the mechanism of uptake of the drug in these parasites (see Section 3.6). Melarsen oxide is taken up very actively and is concentrated by the trypanosomes. Probably, in this way the concentration of the free active agent is increased within the glycolytic compartment, ensuring a complete inhibition of the targets even at low extracellular concentrations of the drug. However other possible alternatives should be discarded in order to substantiate the one mentioned above.

3.3.3 The effect of melarsen oxide on the glucose consumption and pyruvate production

Another two parameters directly related to the general metabolism of trypanosomes are the consumption of carbohydrate and the production of pyruvate. Figure 3.11a shows the results of a series of experiments, when the inhibition of glucose utilisation is studied as a function of the concentration of melarsen oxide. The percentage inhibition represents the figures compared with non-treated cells for both lines of the parasite. It may be seen that there are again different patterns for parent and resistant line. The $I_{50}$ value for the parent line was approximately $6.0\text{-}6.5 \times 10^{-6}$ M melarsen oxide and the maximal inhibition obtained for the resistant line was 40% at the highest concentration of the drug used ($250 \times 10^{-6}$ M). As for the possible mechanism of inhibition, several alternatives have been suggested; first a site of inhibition between

glucose-6-phosphate and triose phosphates (Flynn, 1971), secondly inhibition of hexokinase (Chen, 1948) and thirdly, the possible inhibition of the transport of carbohydrates into the cells. These alternatives are discussed briefly at the end of this section.

Figure 3.11b shows the pattern of inhibition of pyruvate formation for the parent and resistant lines. Again a major difference is found between the two lines. The $I_{50}$ value for the inhibition of the parent line is approximately 3.5-4.0 x $10^{-6}$M melarsen oxide and only 40% inhibition for the resistant line is found at the highest concentration of the drug (250 x $10^{-6}$M).

3.3.4 Inhibition of oxygen and glucose consumption and pyruvate production in parent and resistant lines

The pattern of inhibition for the three metabolites investigated in whole cells are presented in the following table:

| Parent line | Metabolic parameter (consumption/production) | | |
|---|---|---|---|
| | $O_2$ | glucose | pyruvate |
| Melarsen oxide $I_{50}$ ($10^{-6}$M) | 6.0-6.5 | 6.0-6.5 | 3.5-4.0 |
| Inhibition at low concentration of melarsen oxide (2.5 x $10^{-6}$M) | 15% | 20% | 30% |

It would appear that the results presented corroborate the view that pyruvate kinase is the major focal point of inhibition by melarsen oxide (Flynn and Bowman, 1974), as suggested by the clear effect of the drug on pyruvate production. Therefore the control situation, which is the behaviour of the sensitive trypanosome under the effect of the drug allows the possibility of treating the data obtained for the resistant line in a similar way.

When comparing the patterns of inhibition for the resistant line, it was not possible to obtain 50% inhibition even at high concentration of melarsen oxide ($250 \times 10^{-6}$M). Therefore the $I_{25}$ values are used for the data obtained using the resistant line.

| Resistant line | Metabolic parameter (consumption/production) | | |
|---|---|---|---|
| | $O_2$ | glucose | pyruvate |
| Melarsen oxide $I_{25}$ ($10^{-6}$M) | 100 | 70 | 25 |
| Maximal inhibition obtained | 25% | 40% | 40% |

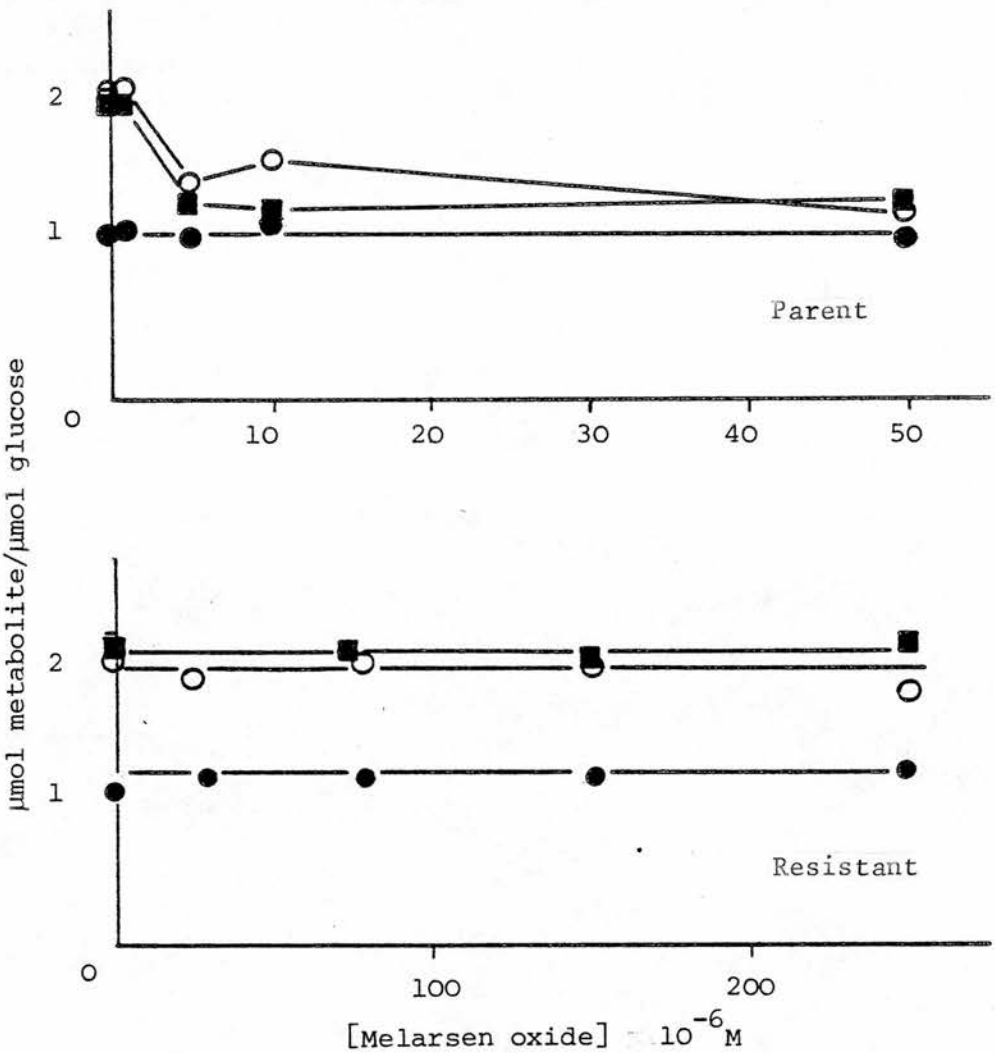
As shown in these data pyruvate production is still a sensitive point as judged by the lower $I_{25}$ value obtained. It is suggested therefore that at high melarsen oxide concentrations, the different alternatives proposed when the inhibition of glucose utilisation was considered may produce a general case of multisite inhibition. However it appears that the focal point of inhibition of pyruvate production is still present in the resistant line.

The sensitivity of pyruvate formation and the other metabolic parameters is only shown at higher extracellular concentrations of the drug in the resistant line. Therefore the possibility of a permeability barrier to the drug in the resistant line is suggested.

3.3.5 The effect of melarsen oxide on the molar ratios of parent and resistant lines

It was found at the beginning of this chapter that the resistant line of T. brucei has a minor difference in its metabolism which was evidenced as a lower production of pyruvate when compared with the parent line. In the two last sections the glucose and oxygen consumption and pyruvate production were found not to be inhibited to the same extent in the two lines, the resistant line being considerably less sensitive

Figure 3.12: The effect of melarsen oxide on the molar ratios with respect to glucose in parent and resistant lines.

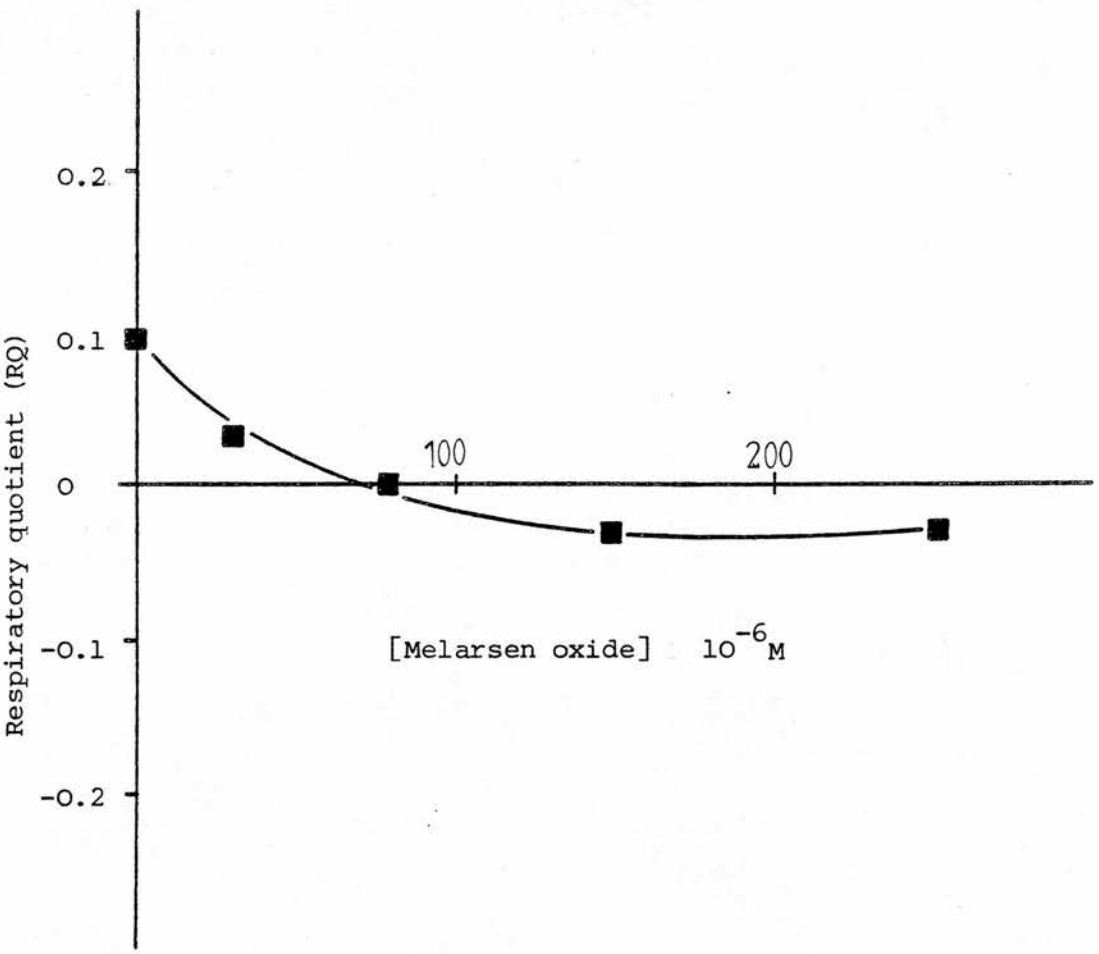



The data collected from the metabolite experiments (Fig. 3.9 and Fig. 3.11) performed under the effect of melarsen oxide were transforme and the metabolites produced or consumed were expressed with respect to the glucose used as a function of the drug concentration. ●-● oxygen/ glucose; ■-■ pyruvate/glucose; O-O pyruvate/oxygen.

to inhibition. The new distribution of metabolites in the resistant line and the differential inhibition of the metabolic parameters did not answer about the question of a possible involvement of a metabolic change as being responsible for the development of the resistant character. A series of experiments were therefore performed in parent and resistant line, with the standard manometric system, in which the behaviour of the metabolic ratios, of parent and resistant line, were investigated as a function of melarsen oxide concentration. Figure 3.12 shows the results obtained. It was observed that in the parent line there was a reduction of the pyruvate/glucose and pyruvate/oxygen ratios from 2 to 1.2 approximately at $5 \times 10^{-5}$ M melarsen oxide. These results show the primary action of melarsen oxide on the production of pyruvate. The data for the resistant line was different, again lower values for the pyruvate/glucose and pyruvate/oxygen ratios being obtained in the absence of melarsen oxide and remaining constant over the range of drug concentration used.

The already suggested possibility of a lack of sensitivity of the trypanosomas as a result of a permeability barrier still remains as an explanation of the molar ratios obtained in both parent and resistant line. However, the possibility that the enzyme pyruvate kinase itself is no longer affected can not be discarded at this stage. Therefore the sensitivity of this enzyme to melarsen oxide in the resistant line became important and it deserves to be studied more carefully in order to corroborate the idea of a permeability barrier. The role of phosphoglycerate kinase, also involved in the phosphorylation of ADP at the substrate level, is potentially important as this enzyme may also participate in the development of the new character in the resistant line; hence these two enzymes are the subject of a more detailed analysis in the next chapter of this thesis.

Figure 3.13: The effect of melarsen oxide on the respiratory quotient in the resistant line.




All experimental conditions are as described in Figure 3.9. For the estimation of $CO_2$ the Direct Method of Warburg was used. Details are as described under Materials and Methods (Section 2.11). The results from duplicate pairs of flasks are expressed as the RQ values as a function of the drug concentration.

3.3.6 The effect of melarsen oxide on the respiratory quotient in resistant line

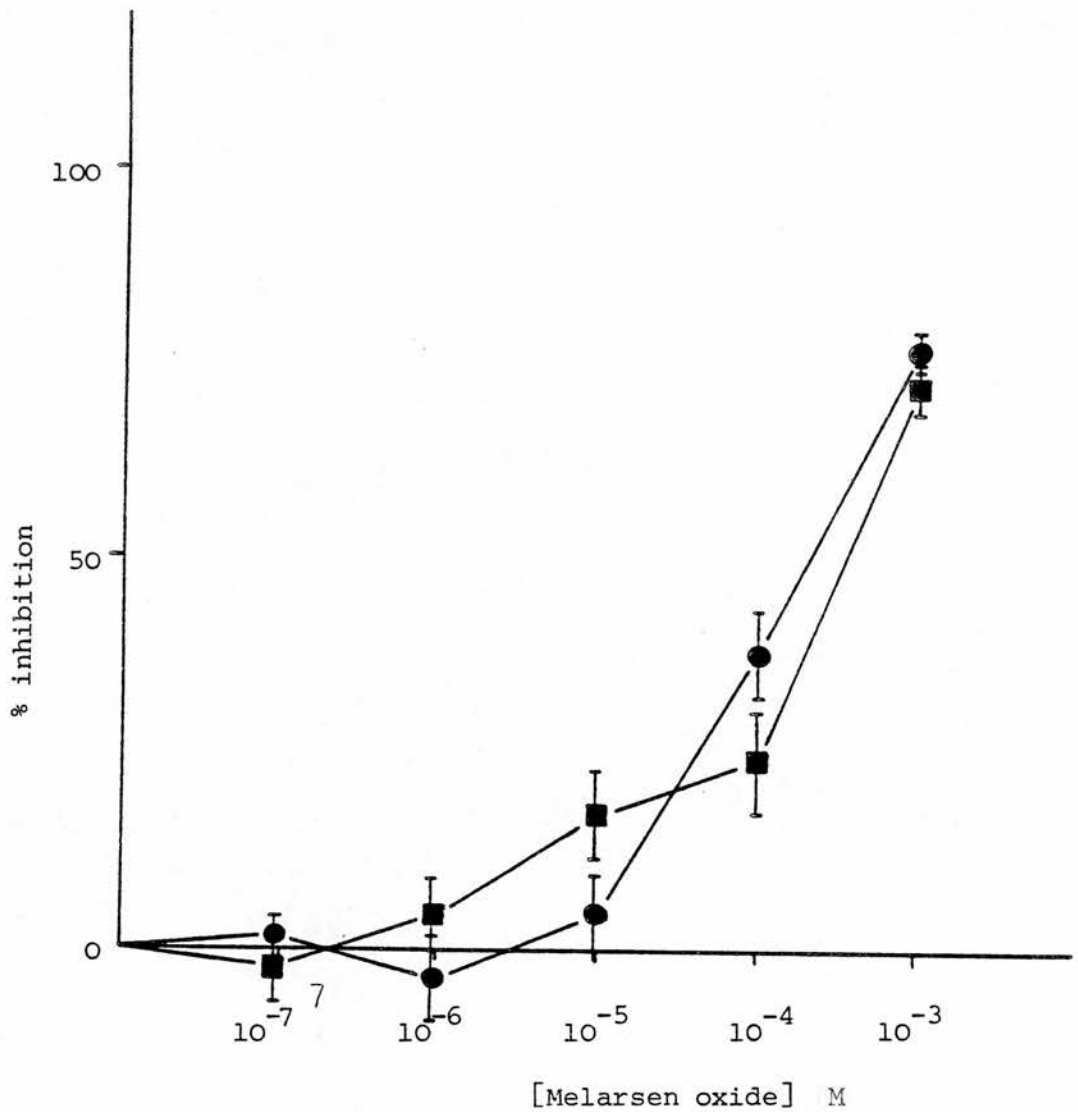
The aerobic metabolism of glucose by LS forms in recently isolated lines of T. brucei produces pyruvate as a major end product, and traces of succinate and $CO_2$ (Ryley, 1956; Flynn and Bowman, 1973). However, upon adaptation to laboratory conditions by syringe passage, it was found that there was a concomitant decrease of the RQ value to less than 0.1 (Flynn and Bowman, 1973). It has been reported in this work (see Table 3.3) that the resistant line developed, produced some $CO_2$ when compared to the parent line. In Figure 3.13 is reported the influence of melarsen oxide on the RQ value in the resistant line. The standard manometric conditions, for the estimation of $CO_2$, were used as described in Material and Methods. It would appear that the $CO_2$ production originally found is inhibited, as are the rest of the metabolic parameters in the resistant organisms, when the drug is present. However the inhibition of $CO_2$ formation is apparently completely abolished at approximately 80 x $10^{-6}$M melarsen oxide.

3.3.7 The effect of melarsen oxide on the multienzyme activity of the glycosome

As described by Oduro (1977) and Opperdoes and Borst (1977), the localisation of some of the glycolytic enzymes in the glycosome allows the particle to catalyse the conversion of glucose or glycerol into 3 phosphoglycerate. At the same time Oduro (1977) described an assay system which relies on the reduction of dehydroxyacetone phosphate to glycerophosphate in the presence of NADH, to measure the multienzyme activity of the system following the decrease in the absorbance at 340 nm (details of the assay medium are given in Material and Methods).

It was considered relevant at this stage to study the sensitivity to melarsen oxide of isolated glycosomes from parent and resistant lines.

Figure 3.14: The effect of melarsen oxide on the multienzyme activity of the glycosome.



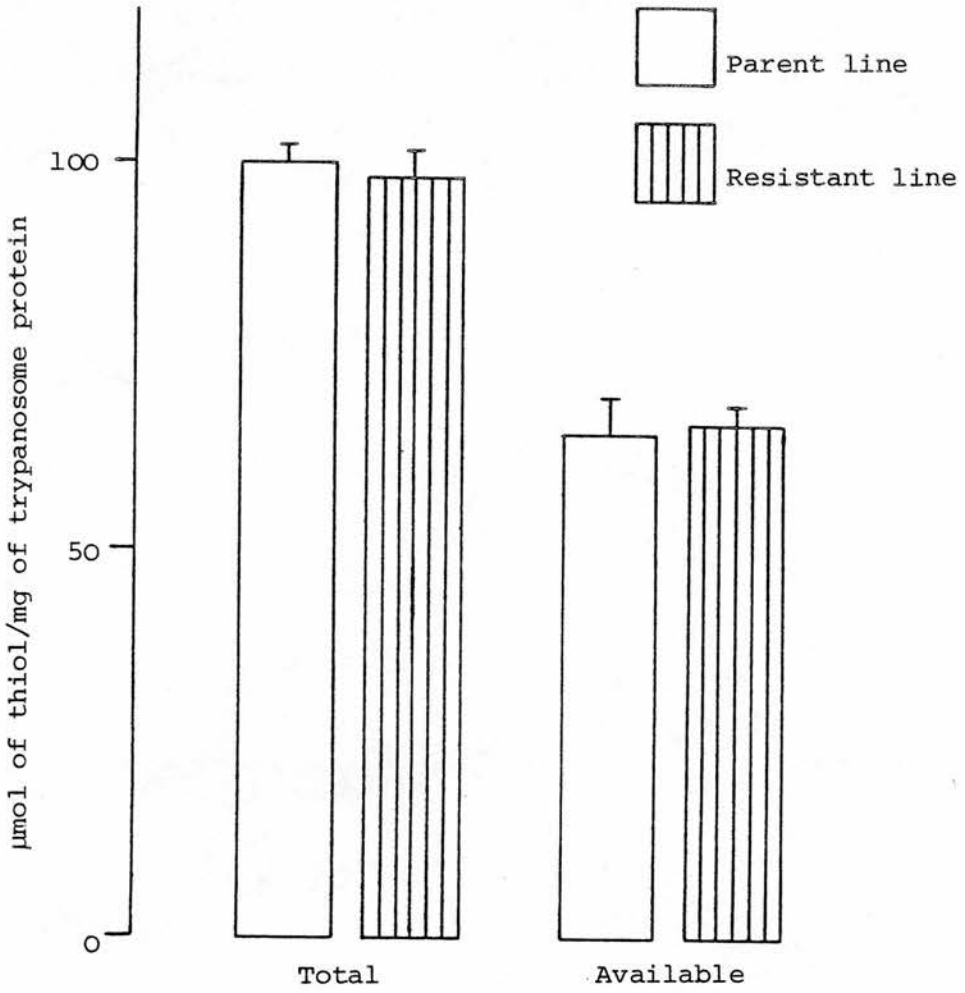

The glycosomes were prepared and assayed from both parent and resistan lines using the techniques described under Materials and Methods (Section 2.14). The glycosomes were preincubated with all the compone of the assay medium including malarsen oxide, for no less than five minutes, and the reaction was started by addition of glucose to the cuvettes. The linear activities were measured about 15 minutes after addition of glucose (see text). The results are presented as percenta inhibition compared to untreated glycosomes as a function of the drug concentration. ●-● parent line; ■-■ resistant line.

The results of duplicates of two sets of experiments are shown in Figure 3.14. It may be seen that although the variability was quite large, there was no inhibition of the multienzyme complex up to the micromolar range of concentrations of the drug. The $I_{50}$ values are approximately 3 to 5 x $10^{-3}$M melarsen oxide for parent and resistant line respectively. No significant differences can be assigned to the values observed. It should be noted, that the reaction was started by addition of glucose to the medium including the drug and the glycosomes from both lines of parasites. A lag phase of activity was observed of up to 15 minutes, before linear traces. This lag phase was interpreted as being a result of substrate diffusion and the time required to reach steady state concentrations of metabolites in the glycosome. No specific activities are presented for the glycosome preparations as it was found that there was a considerable variation from batch to batch of glycosomes prepared from either line of trypanosome, a feature already reported by Oduro (1977). The similar patterns observed suggest that if there is any change in this part of the glycolytic pathway as a consequence of the development of resistance, it is not detectable under the conditions used. However, the possibility of changes in the kinetic properties and sensitivities of isolated components can not be eliminated.

3.3.8 Determination of thiol content in parent and resistant lines

When Voegtlin et al. (1923) postulated their hypothesis of thiol groups (-SH) as the chemoreceptors for arsenicals, the view that an excess of -SH groups will detoxify these drugs in a resistant line was suggested (Voegtlin et al., 1924). The hypothesis has been tested at different times and no differences have been found (Hawking, 1938; Harvey, 1949). In spite of the information gathered, it was necessary to check the -SH group content of the newly developed resistant line of T. brucei.

Figure 3.15: The estimation of thiol content in trypanosome protein from parent and resistant lines using the Ellman reagent




Lyophilised protein from both lines of T. brucei was resuspended in 0.08M phosphate buffer pH 8.0. Available and total thiol (in buffer +/- 2% SDS) were estimated by addition of an aliquot of DTNB solution followed by incubation for 15 minutes. The colour development was then measured at 412 nm. For details see the text and Materials and Methods (Section 2.21).

The reagent 5,5 dithiobis (2-nitrobenzoic acid) (DTNB) originally described by Ellman (1959) was used to estimate the -SH group content of parent and resistant lines. The routine of Habeeb (1972), which estimates available and total -SH group, was used. The data shown in Figure 3.15 suggests that there is no difference in the -SH content, either as available -SH groups or when the protein is denatured (total -SH groups). The detection of more -SH groups upon denaturation and unfolding of the proteins is probably due to the release of hidden groups with original steric impediment and hence no reactivity for DTNB.

3.3.9 Summary

It has been suggested earlier that there was a possibility of explaining the development of resistance to arsenicals, in terms of a metabolic change in the trypanosomes resulting in a bypass of the arsenical sensitive site. This hypothesis is not by any means accepted by all the authors in the field but has been considered as a viable alternative. Therefore this suggestion was experimentally tested. The general carbon balance was first studied in the absence of drug, in both lines of parasite. The characteristics found for the parent line agreed with the data already reported for other laboratory adapted LS form of trypanosomes of the brucei group. However, the metabolism of the resistant line was apparently modified, the differences found being lower pyruvate/glucose and pyruvate/oxygen ratios (1.73 and 1.84 respectively). A small production of $CO_2$ resulting in an RQ of approximately 0.1 was also found with the resistant line.

The metabolism of both lines was also studied in the presence of melarsen oxide. As reported in the development of the resistant line the oxygen consumption was inhibited to a lesser extent in the resistant line. This information was extended and it was found that glucose consumption and pyruvate production are also less inhibited in the resistant line.

The idea of a permeability barrier for the drug, as an alternative to explain the development of resistance, emerged in two different ways. First, the different patterns for the oxygen uptake in whole cells of parent and resistant line when compared with the similar pattern of inhibition of the same metabolite in water lysates from both lines. Secondly it was found that resistant trypanosomes are still sensitive to melarsen oxide but at relatively higher concentrations of extracellular drug.

As for the importance of the lower levels of pyruvate obtained and the $CO_2$ production in the resistant line, it appeared that in the case of the molar ratios, they were conserved during the exposure of the resistant line up to a concentration of $250 \times 10^{-6}$M melarsen oxide. The $CO_2$ formation was inhibited to such an extent that $CO_2$ was no longer detectable under the experimental conditions used. It is suggested that although the modification in the resistant line may be the result of further metabolism of pyruvate possibly involving decarboxylation, the metabolic difference itself does not participate directly in the development of resistance. Another alternative, the possible excess of thiol groups as detoxifying agents, was tested; no significant differences were found between parent and resistant line.

## 3.4 Enzymological studies

From the metabolic studies it was suggested that although an apparent modification of metabolism is present in resistant line, this modification does not account for the development of resistance to the drug. Furthermore on the inhibition of the evolution of the different metabolites in both lines suggested the possibility of a permeability barrier to the drug as a result of the development of resistance. This suggestion, however, does not rule out the alternative of modification of the enzyme targets to melarsen oxide. Therefore a closer look was taken at pyruvate kinase, a known target for the drug, and to phosphoglycerate kinase, an enzyme with a similar function. The experiments performed covered the possibility of increased production of the enzymes as a mechanism to overcome the action of the drug and also the possibility of enzyme modification, either producing different kinetic characteristics or different sensitivities to the drug.

Finally, as suggested in the Introduction, phosphoglycerate kinase is the least studied enzyme of the glycolytic kinase group in trypanosomes. Therefore some experiments were performed to study the general properties of the enzyme, mechanism of action and mechanism of inhibition by arsenicals.

### 3.4.1 Choice of materials and optimal conditions for assay

Two assay systems were used throughout the experimental part of the enzymological studies. Pyruvate kinase (PK) was assayed by the method of Bücher and Pfleiderer(1962) as modified by Flynn (1971). This system was used recently in the purification of PK from T. brucei (Flynn and Bowman, 1980). Phosphoglycerate kinase (PGK) was assayed by the method of Krietsch and Bücher (1970). The system, as described in Material and Methods, relies on the back reaction catalysed by PGK coupled to the oxidation of NADH by glyceraldehyde-3-phosphate dehydrogenase. The reaction is followed spectrophotometrically as a decrease in the absorbance at 340 nm.

3.4.2 The source of enzymes used

The source of enzymes used in the experiments was lyophilised material prepared immediately after lysis of purified trypanosomes in distilled water. The material from either resistant or parent line was pooled and stored *in vacuo* at low temperature (2-4°C). The specific activity of PGK varied slightly from batch to batch (0.7-1.0 μmol $min^{-1}$ $mg\ protein^{-1}$). It was found that under the conditions specified above, the activities showed no considerable reduction for a period of up to six months. Lactate dehydrogenase in the lyophilised material is regarded as evidence of contamination from host cells. Thus any batch with measurable traces of this enzyme was discarded.

3.4.3 The buffer system and experimental conditions for assay

a) Pyruvate kinase

The buffer characteristics and conditions used for PK have been already described (Flynn, 1971). Detail of the assay medium are presented in Material and Methods.

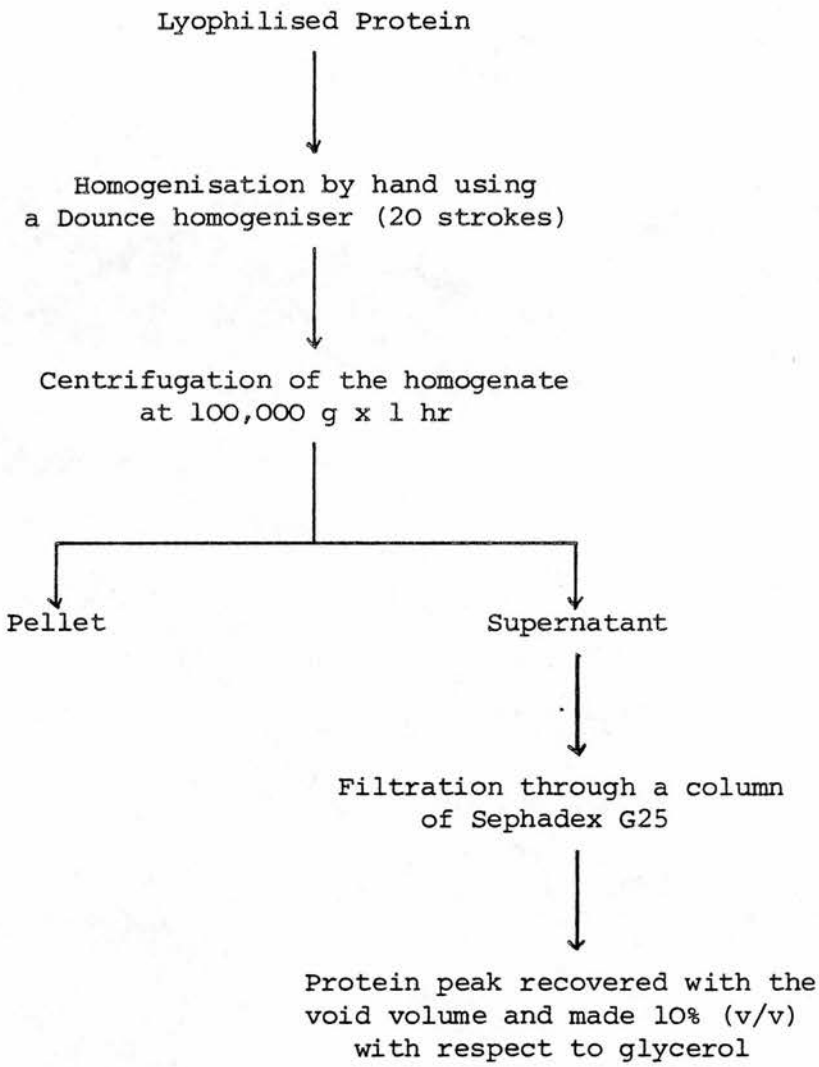
b) Phosphoglycerate kinase

The conditions originally described by Bücher (1955) used phosphate buffer for the evaluation of the physicochemical parameters of the enzyme. However it was found that using triethanolamine (TEA) or tris-hydroxymethyl-aminomethane (Tris) a higher activity (by a factor 2-3) was observed when different buffers where compared over a wide range of pH values. As TEA produced the most consistent higher results it was chosen as the buffer of choice in the standard assay system. Other conditions for the assay of PGK are described in next sections of this chapter.

3.4.4 The preparation of enzymes for kinetic studies

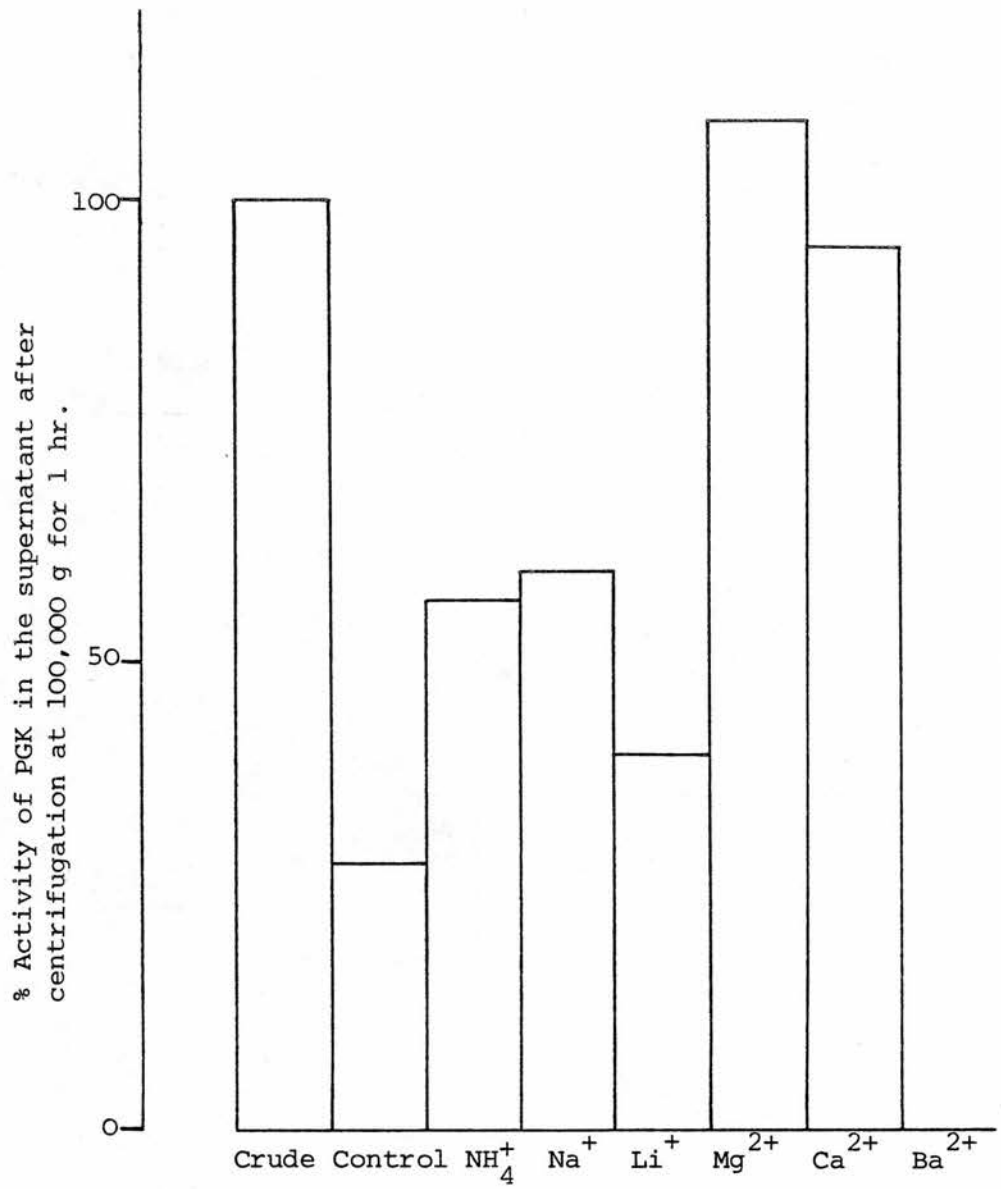
As reported in the last section, the freeze dried material was used as a source of enzyme in the experiments performed. The homogenates produced after resuspension of the dried material were cloudy and heterogeneous

Figure 3.16: Semipurification scheme used for the preparation of enzymes for the enzymological studies.




Lyophilised protein from both lines of T. brucei was resuspended in the appropriate buffer (see text for details) and hand homogenised in an ice bath. The purification scheme was followed as above. The Sephadex column with a bed volume of 20 ml was previously equilibrated with the buffer as mentioned above. The protein peak was collected with the void volume of the column and diluted with glycerol.

Figure 3.17: The effect of divalent cations on the solubilisation of trypanosome phosphoglycerate kinase.




1 mg/ml of lyophilised protein from parent line was resuspended in TEA buffer pH 7.5 (see Section 2.3) containing 0.2M of the different salts mentioned in the figure, in the chloride form. The suspension was homogenised by hand in an ice bath. The rest of the experimental conditions were as described in Materials and Methods (Section 2.9). For assay of activity of the enzyme see Section 2.10.2. Crude = suspension without centrifugation; control = suspension centrifuged without salt.

and may have been contaminated with endogenous nucleotides and coenzymes. Therefore a semipurification scheme was derived to prepare the enzymes for kinetic studies.

For the purification of PK, the buffer conditions described by Flynn (1971) were used (Tris 0.1M, $10^{-3}$M DTT, pH 7.2 with KOH/NaOH). Hence 50 mg of freeze dried material was resuspended in 5 ml of buffer and submitted to the partial purification scheme in Figure 3.16.

The supernatant obtained after high speed centrifugation was desalted using a column of Sephadex G25 (20 ml bed volume). The main protein peak collected with the void volume was made 10% (v/v) with respect to glycerol and used for kinetic analysis.

The situation however was not the same for PGK as the major enzymic activity was found in the pellet after high speed centrifugation. Attempts were made to solubilise the enzyme with Triton X-100 and various salts. The latter were found to produce more consistant results. Therefore the effect of different cations, in the chloride forms, was investigated on the possible solubilisation of PGK and subsequent recoveries of the activity in the supernatant after high speed centrifugation. Figure 3.17 shows the results obtained. It is observed that $Mg^{2+}$ and $Ca^{2+}$ ions produced the highest soluble recoveries when compared to the crude material and the supernatant without added salts. Some residual activity was always found in the pellets. The barium salt produced complete loss of activity at the concentration used (0.2M). Due to the yield obtained with the magnesium salt it was chosen as the standard constituent of the buffer for the purification of PGK.

The rest of the purification scheme was similar to the one proposed for the semipurification of PK including the desalting stage. The buffer used was 10 x $10^{-3}$ M TEA, pH 7.5 containing $10^{-3}$M DTT. When inhibition studies were performed with the enzymes the sulphydryl agent was excluded from the preparation.

3.4.5 Preliminary experiments on the purification of phosphoglycerate kinase

As discussed earlier in this chapter the possibility of misleading results is always present when the kinetic and inhibitory experiments are done with relatively impure enzymes. Although it has been reported that the general characteristics of pyruvate kinase from T. brucei were not altered to a great extent with the purification procedure (Flynn, 1971), it was considered that this may not be the general situation and therefore a purification scheme was investigated.

The classical approach was taken in order to purify the enzyme PGK and then perform the experiment required to establish comparisons, not only with the impure material but with the enzyme in the mammalian model.

The following methods of purification were evaluated:

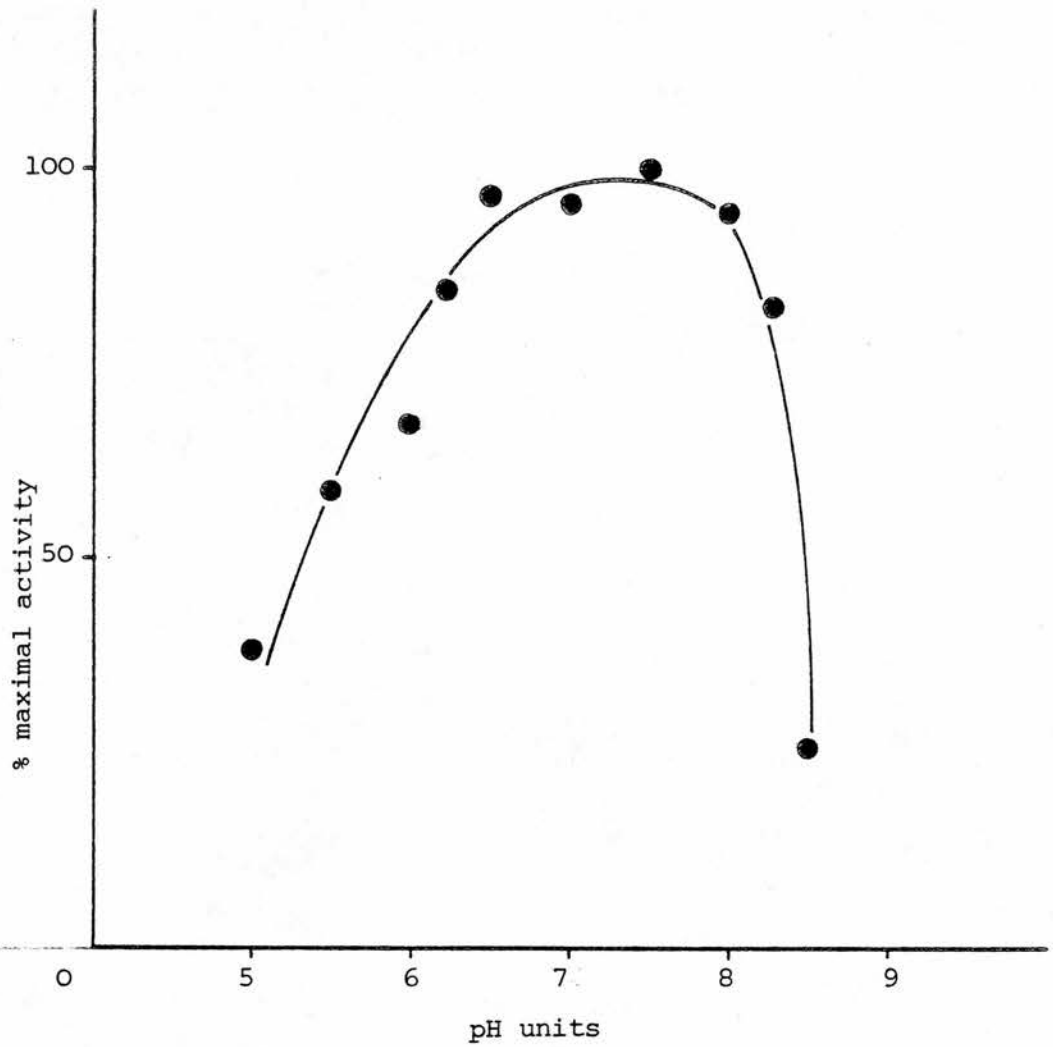
- Neutral salt precipitation
- Detergent solubilization
- Gel filtration: G100
  G200
  Biogel 0.5M
  Sepharose 4B
- Ion exchange chromatography: CM 52
  DEAE 52
- Affinity chromatography: Blue Sepharose
- Hydrophobic chromatography: Octyl sepharose
  Phenyl sepharose

Promosing results have been obtained with some of the techniques used. However a final purification scheme still remains to be worked out.

3.4.6 The effect of pH on the activity of phosphoglycerate kinase

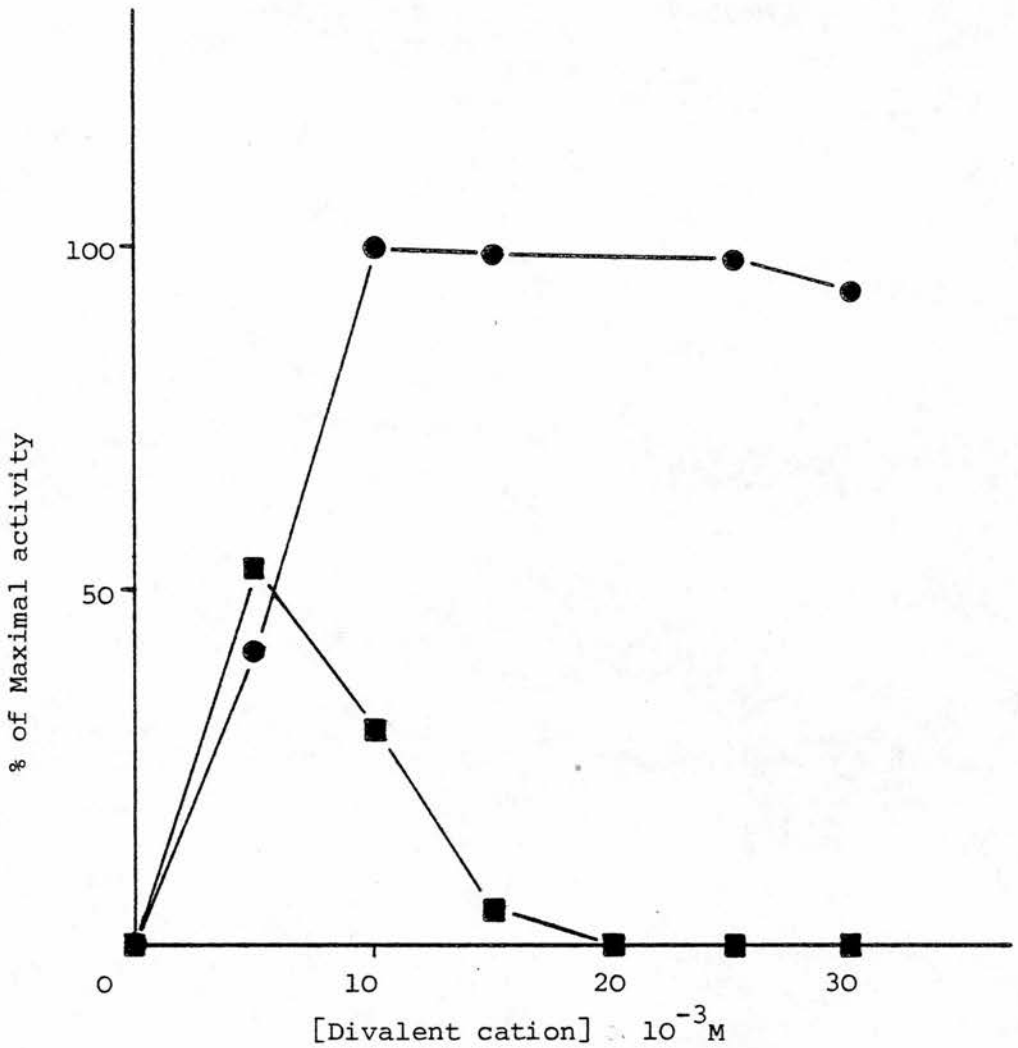
For the determination of the effect of pH, three buffer systems were used. The range of pH investigated was from 5.0 to 8.5. First the

Figure 3.18: The effect of pH on the activity of phosphoglycerate kin




Enzyme aliquots were prepared as in Section 2.9 and assayed in the following buffers systems: 0.1M Tris, pH from 7.0-8.5; 0.1M triethanolamin pH from 5.0-8.0; and a mixture of the following buffers, 0.03M acetat 0.02M histidine, 0.01M imidazole, 0.01M Tris and 0.03M glycine, pH from 5.0-8.5. The rest of the components, except the buffer and condition for assay, are as described under Materials and Methods (Sedtion 2.10.2). For the presentation of results see text.

Figure 3.19: The effect of divalent cations on the activity of phospho glycerate kinase.




Enzyme samples were prepared and estimated as described under Materia and Methods (Sections 2.9 and 2.10.2). The different cations at the us concentrations were also present in the assay medium before addition o the enzyme sample. The results are presented as the percentage of the enzyme activity for each cation as a function of the concentra

●–● $Mg^{2+}$; ■–■ $Mn^{++}$.

Enzyme activity with $Mg^{2+}$ ($10 \times 10^{-3}$M)= 100%

activity was estimated in the range from 7.0-8.5 using Tris buffer, second from 5.0-8.0 in TEA and third in a mixture of the following buffers: 0.03M acetate, 0.02M histidine, 0.01M imidazole, 0.01M Tris, 0.03M glycine.

The values obtained with the three buffer systems showed some variability. Therefore to obtain comparable results a value of 100% was assigned to the highest value estimated with each buffer and the rest of the activities were compared with this maximal value. Figure 3.18 shows that the apparent optimum pH lies between 6.5-8.1. A sharper decrease in activity at the alkaline end of the scale was observed. A pH of 7.5 was selected as the standard value for the assay of PGK.

3.4.7 The effect of divalent cations on phosphoglycerate kinase activity

PGK from other sources has a requirement for divalent cations ($Mg^{2+}$, $Mn^{2+}$) for activity. It has also been suggested that the true substrates of the reaction, either in the backward or forward direction, are ATP or ADP complexed with these ions, preferably $Mg^{2+}$ (Larsson-Raznikiewicz, 1964; 1967; Rao and Osper, 1961). Figure 3.19 shows the response of PGK to two cations ($Mg^{2+}$ and $Mn^{2+}$) added as the chloride salts. Their influence on the activity seems to be in two phases. First a rapid activation of the enzyme, the concentration required to give maximal activity being $5 \times 10^{-3}$M and $10 \times 10^{-3}$M of $Mn^{2+}$ and $Mg^{2+}$ respectively. Secondly a region of inhibition which was found more noticeable in the case of $Mn^{2+}$ than with $Mg^{2+}$. Similar results were found when the metal ion specificity was studied in PGK from yeast (Larsson-Raznikiewicz, 1970a; Rao and Osper, 1961). A possible reason for the inhibition by $Mn^{2+}$ is its uncompetitive inhibition effect at concentrations higher than $1 \times 10^{-3}$M (Webb, 1963). The $Mg^{2+}$ salt was chosen, at a concentration of $10 \times 10^{-3}$M, for the standard assay conditions of PGK.

Table 3.6: The nucleotide specificity of semipurified trypanosome phosphoglycerate kinase.

| Nucleotide | % Activity | $K_m$ ($10^{-3}$ M) | $V_{max}$ units mg protein$^{-1}$ |
|---|---|---|---|
| ATP | 100 | 0.89±0.22 | 0.90±0.18 |
| CTP | 11.1 | 1.65±0.50 | 0.11±0.02 |
| UTP | 15.1 | 3.74±0.76 | 0.14±0.05 |
| ITP | RA | - | - |
| GTP | RA | - | - |

RA = Residual activity only.

Activities are quoted with respect to ATP = 100%.

The kinetic parameters for each nucleotide were determined as in Figures 3.21 and 3.22. The values were obtained from three duplicated experiments with three different enzyme batches. The values are presented as the mean ± standard error of the mean.

3.4.8 The effect of different nucleotides on phosphoglycerate kinase activity

The specificity of PGK for the phosphate donor was determined by replacing ATP with different nucleoside triphosphates. Table 3.6 shows that besides ATP, CTP and UTP could support the activity of the enzyme although at considerably lower activities. It was also found that the enzyme had a lower affinity for those nucleotides. ITP and GTP only showed residual activities at equivalent concentrations to ATP. It is suggested that according to the levels of activity and to the affinities found for the different nucleoside triphosphates, trypanosomal PGK is rather specific in its requirement for a phosphate donor.

Variable results have been obtained with respect to the nucleotide specificity of PGK from other sources (Krietsch and Bücher, 1970; Rao and Osper, 1961). It has been suggested that the order of reactivity is ATP>ITP>GTP>dGTP>dATP. The reactivity of ITP and GTP after ATP has been corroborated by Lee and O'Sullivan (1975); however, the results of this experiment showed that these two nucleotides only supported activity at residual levels.

As a result of the experiments performed in the last sections an assay system was developed for the estimation of trypanosomes PGK activity. The basic components were: TEA buffer at pH 7.5 and $Mg^{2+}$. Further detail on the concentration of substrates and other components are found under Material and Methods.

Table 3.7: The kinetic parameters of pyruvate kinase from parent and resistant lines.

| | | FDP | $S_{50}$ ($\times 10^{-8}$ M) | $V_{max}$ ($\mu$mol $min^{-1}$ $mg\ protein^{-1}$) | Hill coefficient |
|---|---|---|---|---|---|
| PEP | Parent | - | $1.60 \pm 0.30$ | $0.70 \pm 0.02$ | $2.16 \pm 0.30$ (3) |
| | | + | $0.84 \pm 0.25$ | $0.78 \pm 0.01$ | $1.35 \pm 0.22$ (2) |
| | Resistant | - | $1.43 \pm 0.12$ | $0.76 \pm 0.09$ | $2.80 \pm 0.53$ (2) |
| | | + | $0.82 \pm 0.10$ | $0.76 \pm 0.06$ | $1.37 \pm 0.01$ (2) |

| | | $K_m$ ($\times 10^{-4}$ M) | $V_{max}$ (Units $min^{-1}$ $mg\ protein^{-1}$) |
|---|---|---|---|
| ADP | Parent | $3.17 \pm 0.22$ | $0.60 \pm 0.08$ (3) |
| | Resistant | $4.93 \pm 1.24$ | $0.94 \pm 0.33$ (3) |

The effect of PEP concentration on semipurified pyruvate kinase was determined by incubating all the components of the assay mixture at 25°C for 5 min with different concentrations of PEP and saturating concentrations of ADP. The reaction was started by addition of an aliquot of enzyme (20 µl) and the initial velocities were measured. For the effect of ADP concentration all the experimental conditions were kept constant but PEP was present in the incubation mixture at a fixed saturating concentration. The mixture contained variable concentrations of ATP and the reaction was started as above. The initial rates were calculated for both substrates. To estimate the kinetic parameters for PEP a non-linear regression programme of the Hill equation was used. For the analysis of the data with respect to ADP a similar non-linear regression programme of the Michaelis-Menten equation was employed. Details of the methods are as described under Materials and Methods (Sections 2.10.1 and 2.25). Values are presented as mean $\pm$ standard deviation; in parenthesis number of analysis.

## 3.5 Comparative studies on pyruvate kinase and phosphoglycerate kinase from parent and resistant lines

### 3.5.1 The kinetic parameters of pyruvate kinase from parent and resistant lines

As suggested in the chapter on the metabolism of parent and resistant lines, there was a clear difference in sensitivity to melarsen oxide between the parent and the resistant line. It has also been reported that the focal point of action of melaminyl arsenicals is the kinases, particularly pyruvate kinase (Flynn and Bowman, 1974), which accounts for most of the inhibition produced by these active agents. Therefore the sensitivity of the kinases in the resistant line, and their kinetic parameters were investigated.

The general kinetic parameters of pyruvate kinase from the parent line, have been recently described in detail for the semipure enzyme (Flynn and Bowman, 1980). It was found that the enzyme is allosteric with respect to one of the substrates, phosphoenol pyruvate (PEP); fructose diphosphate (FDP) is a heterotropic activator. The characterisation of the enzyme from both parent and resistant line is clearly necessary as a preliminary approach to the description of any possible differences arising as a result of the development of drug resistance.

Using standard assay conditions (see Material and Methods) the system was preincubated for 3 minutes at 25$^{o}$C prior to initiation of the reaction by the addition of enzyme. The kinetic parameters obtained for the enzyme from both lines, in the presence and absence of FDP, are shown in Table 3.7. These data were obtained from three different experiments with three different enzyme preparations. The initial velocities were obtained from at least eight different substrate concentrations, the other cosubstrate being kept at a constant concentration. Palindromic series were used when estimating the initial velocities.

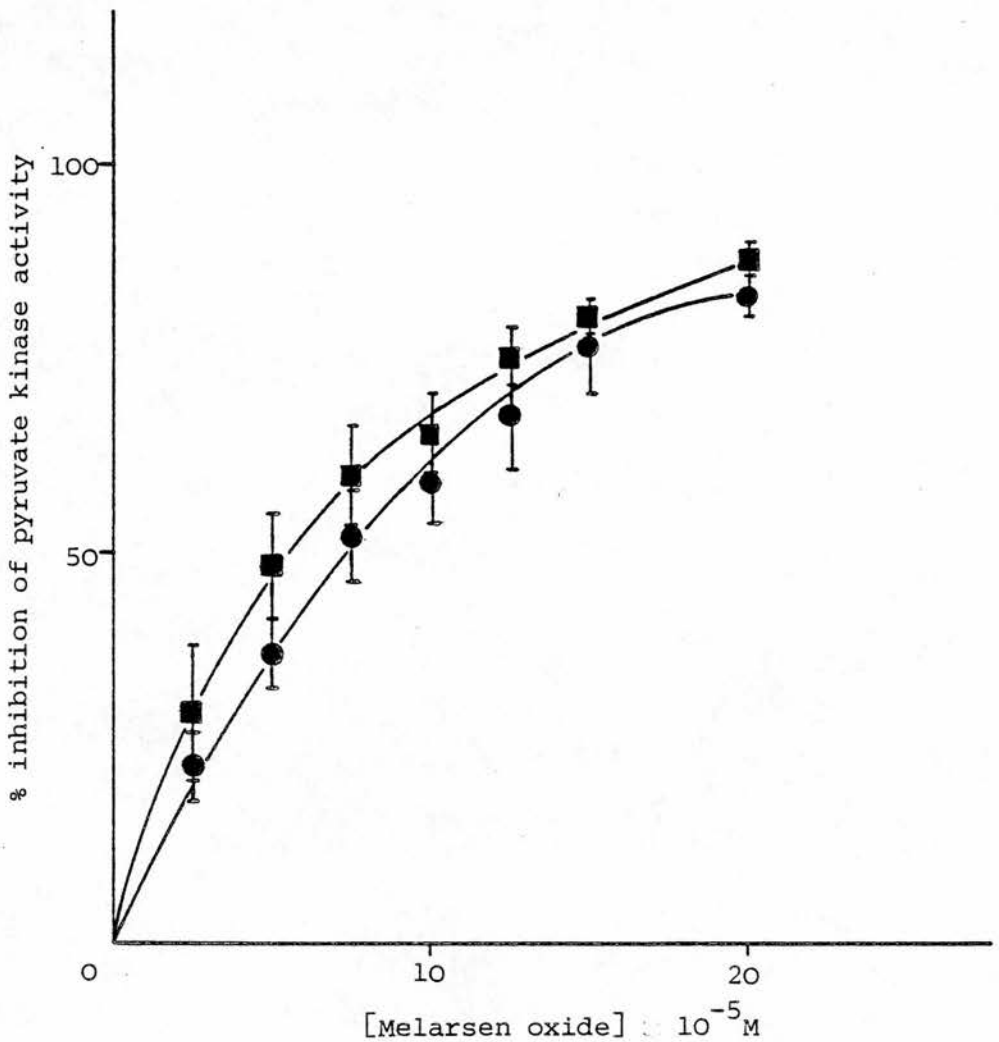
Two different computer programs were used to estimate the kinetic parameters; for the Michaelis-Menten type kinetics (Atkins and Gardner, 1977) and for the Hill-type kinetics (Atkins, 1973).

After a close examination of the data using the paired "student" test, it was found that there were no significant differences between the two enzymes from different lines of trypanosomes, with regard to the $S_{50}$, $K_m$ and $V_{max}$ values for PEP and $K_m$ and $V_{max}$ values for ADP. However a possibly significant difference was found in the "n" value (Hill coefficient) for the enzyme in the absence of FDP (the "t" value found was 5.06 for 2 degrees of freedom). This difference in the interaction coefficient is difficult to evaluate as this parameter has no real biological significance, as suggested recently by Cornish-Bowden (1979) and Atkins (1973), and originally by Hill (1910). With regard to the absolute difference between the two "n" values in parent and resistant line, it is suggested that this may be due to experimental variation in terms of storage and age of the enzyme (Flynn, personal communication).

Another point which made this higher value of "n" of limited interest is the argument that the sigmoidal response of pyruvate kinase with respect to phosphoenolpyruvate is possibly of little physiological importance *in vivo*, as the enzyme in *T. brucei* is suggested to be fully activated by FDP (Flynn and Bowman, 1980) and the value of "n" at these conditions did not show any difference under the experimental conditions used.

The specific activities of the enzymes in term of the $V_{max}$ value ($\mu$mol $min^{-1}$ mg $protein^{-1}$) were the same for the parent and resistant line. Therefore the possibility of a more active enzyme, capable of overcoming inhibition by the drug, is ruled out.

Figure 3.20: The effect of melarsen oxide on the activity of pyruvate kinase.




Enzyme samples were prepared and estimated as described under Material and Methods (Sections 2.9 and 2.10.1). Aliquots of the enzyme were preincubated for 5 minutes with different concentrations of melarsen oxide and the components of the assay mixture. The reaction was started by addition of ADP ($0.4 \times 10^{-3}$M) and PEP ($1.67 \times 10^{-3}$M). The results are presented as percentages of inhibition compared to the uninhibited enzyme as a function of the drug concentration. ●-● parent line; ■-■ resistant line.

3.5.2 The inhibition of pyruvate kinase from parent and resistant lines

From the experiments performed in the last section it is suggested that the kinetic parameters of pyruvate kinase are not affected as a result of the development of resistance in T. brucei. However, this did not rule out the possibility of different sensitivities of the enzyme from the two parasites, to the active agent melarsen oxide.

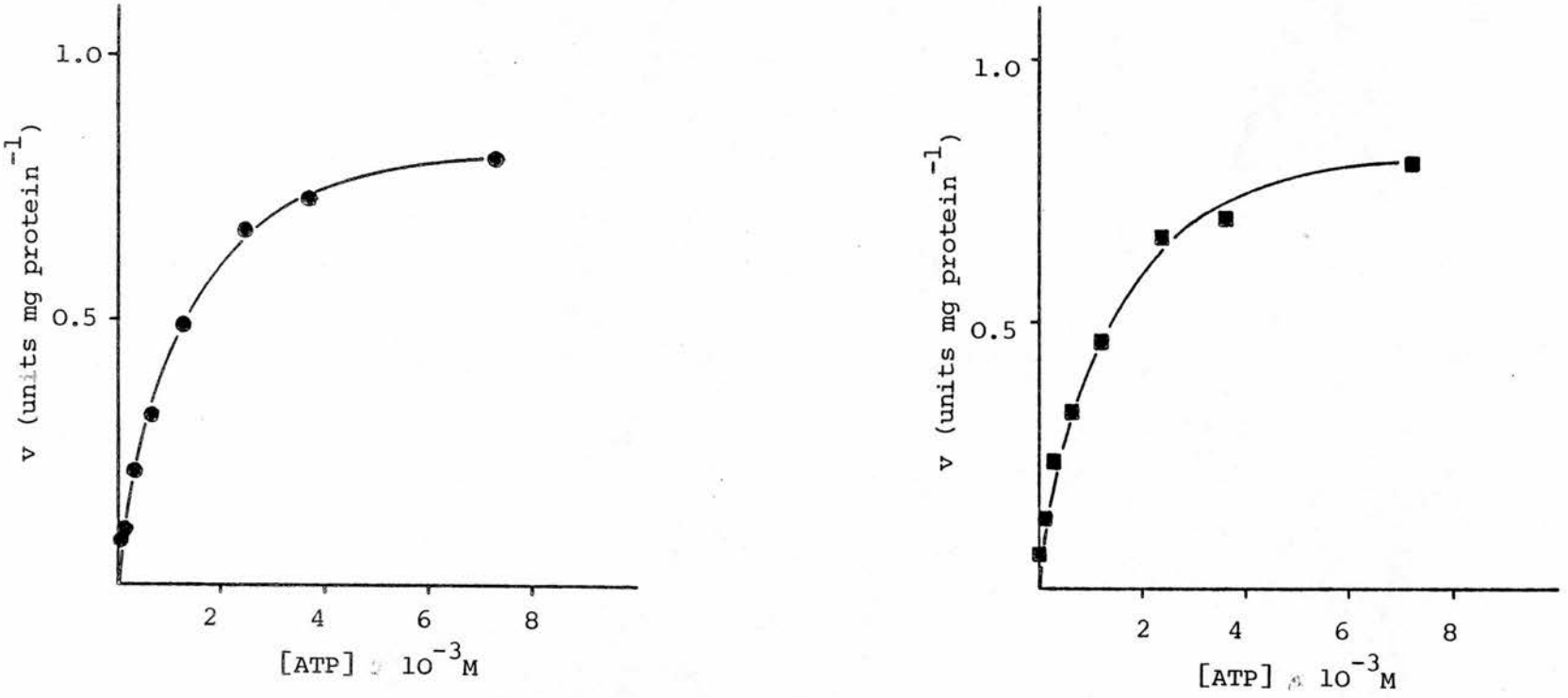
One minor disadvantage of the inhibition studies at this level is the state of purification of the enzyme and the known mechanism of action of arsenicals as thiol poisons. The concentration of drug required to produce a certain inhibition level will be overestimated, as impure sources of enzyme may have a relatively large concentration of free thiol groups (see Section 3.3.8) other than the ones situated on the enzyme. The enzyme from both lines of parasites, have here been taken to the same stage of semipurification.

The mechanism of inhibition by melarsen oxide has been reported previously for this enzyme (Flynn, 1971). It was found that the drug binds competitively with respect to PEP to produce the lethal effect. This characteristic is used in the design of the inhibition experiment (see below).

Figure 3.20 shows the inhibition pattern of PK from parent and resistant lines as a function of the concentration of melarsen oxide. When performing the inhibition studies the enzyme was incubated with the constituents of the standard assay medium and the drug for 5 minutes; the reaction started by addition of PEP and ADP.

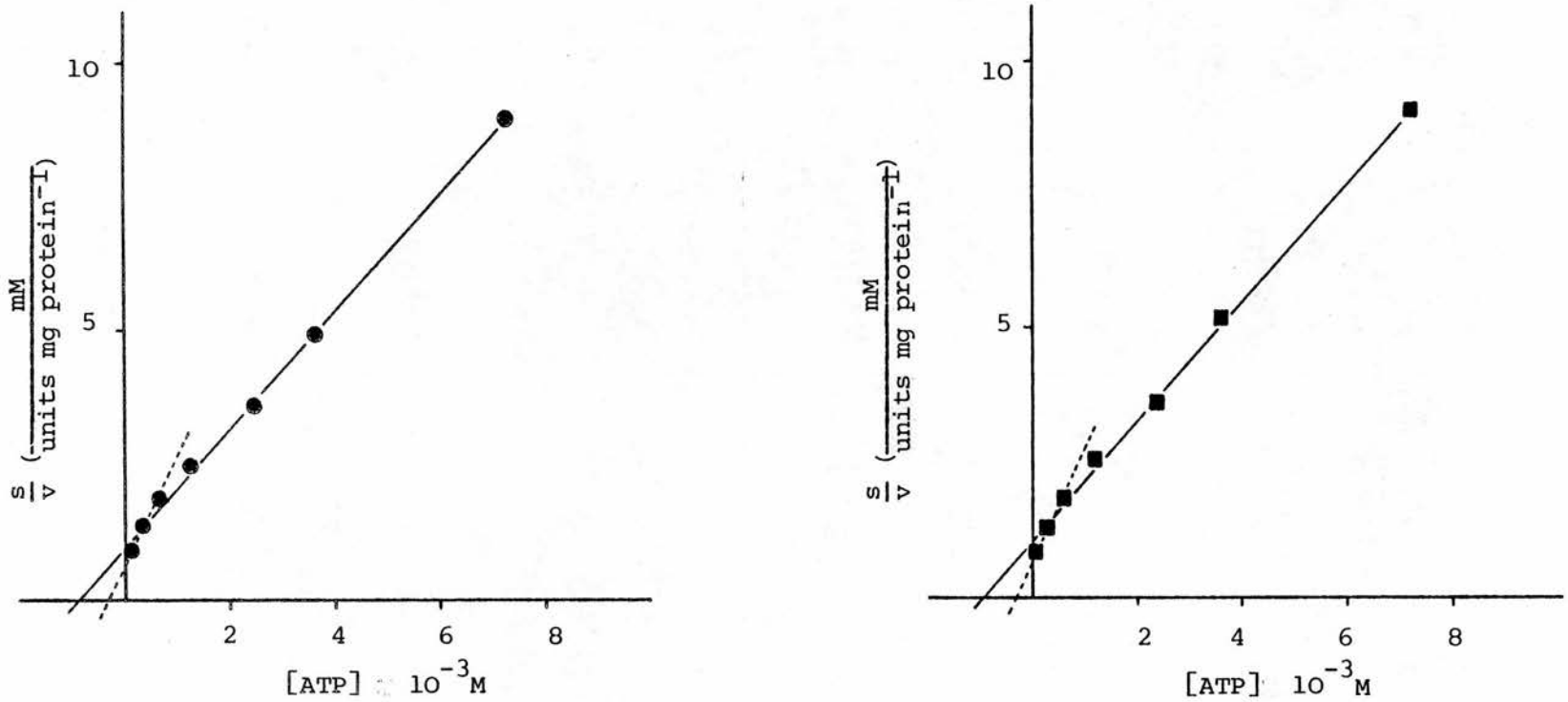
The $I_{50}$ values were apparently the same for both lines with an approximate value of $7.5 \times 10^{-5}$ M melarsen oxide. It should be noticed that each point in the figure represents the mean value of three different experiments with three different preparations of enzymes; the palindromic system was used throughout and FDP was not present in the incubation medium.

Figure 3.21: The effect of ATP concentration on the activity of semipurified phosphoglycerate kinase from parent and resistant lines.




Enzyme samples were prepared and estimated as described under Materials and Methods (Sections 2.9 and 2.10.2). The components of the assay mixture were incubated for 5 minutes with the different concentrations of ATP (G3P constant at 10 x $10^{-3}$ M). The reaction was started by addition of an aliquot of enzyme. The results are presented as the enzyme activity as function of the ATP concentration. ●-●, parent line; ■-■, resistant line.

Figure 3.22: The effect of ATP concentration on the activity of phosphoglycerate kinase: Hanes plot.




The data of Figure 3.21 are derived using the Hanes plots. Linear regression of the data was carried out as described in Materials and Methods (Section 2.24). ●-● parent line; ■-■ resistant line.

It is suggested from the experiments performed on the inhibition of PK that there was no significant differences in the sensitivities of pyruvate kinase to melarsen oxide as a result of the development of resistance.

3.5.3 Kinetic parameters of phosphoglycerate kinase from parent and resistant lines

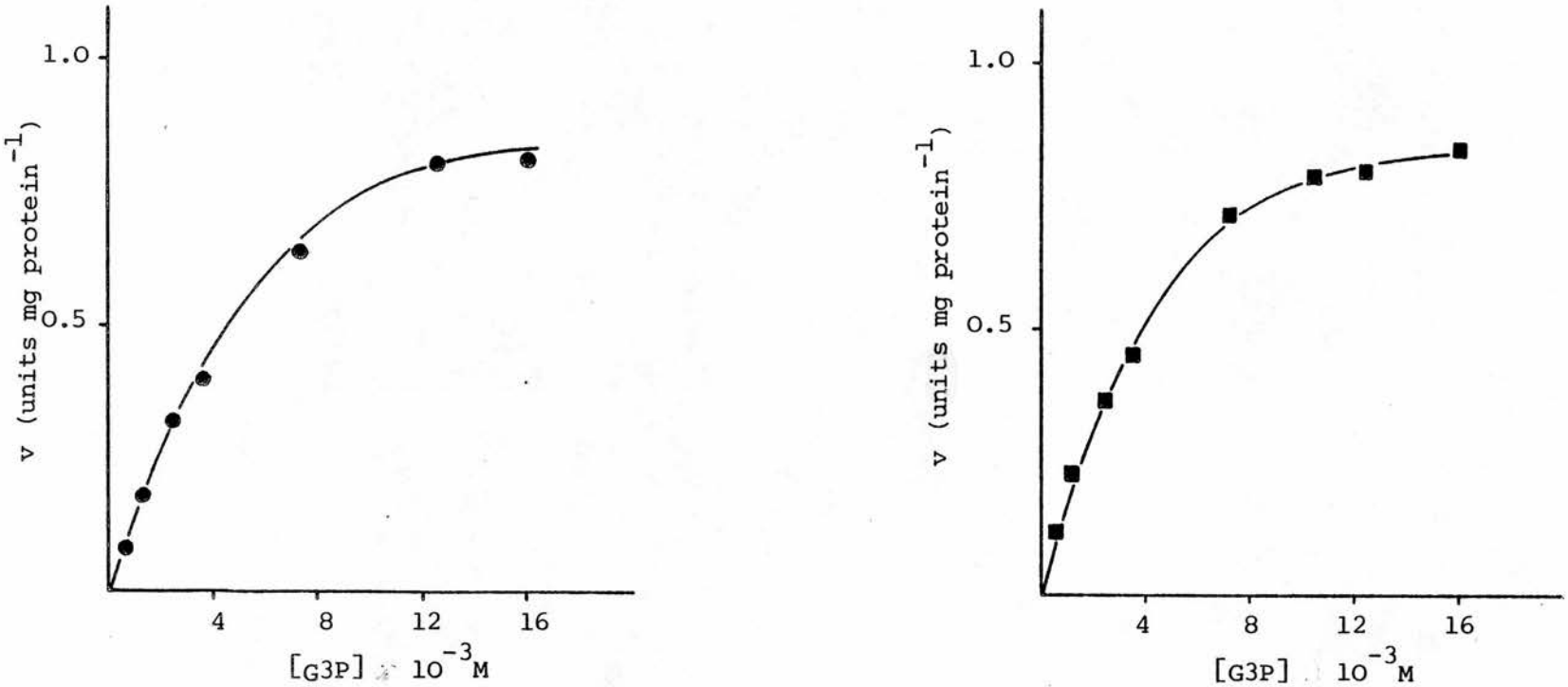
As reported in the Introduction little or no information exists on the general physicochemical parameters of trypanosomal PGK. Nonetheless the enzyme is a key component in the glycolytic sequence of trypanosomes because of its localisation in the glycosome (Opperdoes and Borst, 1977; Oduro, 1977). If the idea of self-sufficiency of this particle is tenable this enzyme should provide the ATP for the phosphorylation of glucose at the beginning of the sequence by hexokinase (see Figure 1.2). At the same time, its possible sensitivity to arsenicals has not been reported and hence its participation in the development of resistance is unknown. As described above, PK a focal point of arsenical action, was not changed in the resistant line. Therefore it was considered relevant to study PGK in more detail.

a. Effect of ATP concentration

After semipurification of the enzyme from both sources as described in an earlier section, the effect of different concentrations of ATP, at a saturating concentration of the cosubstrate G3P, was studied. It should be pointed out that the concentration of $Mg^{2+}$ was in excess of the maximal concentration of ATP in order to ensure that most of the nucleotide is complexed to the ion ($MgATP^{2-}$).

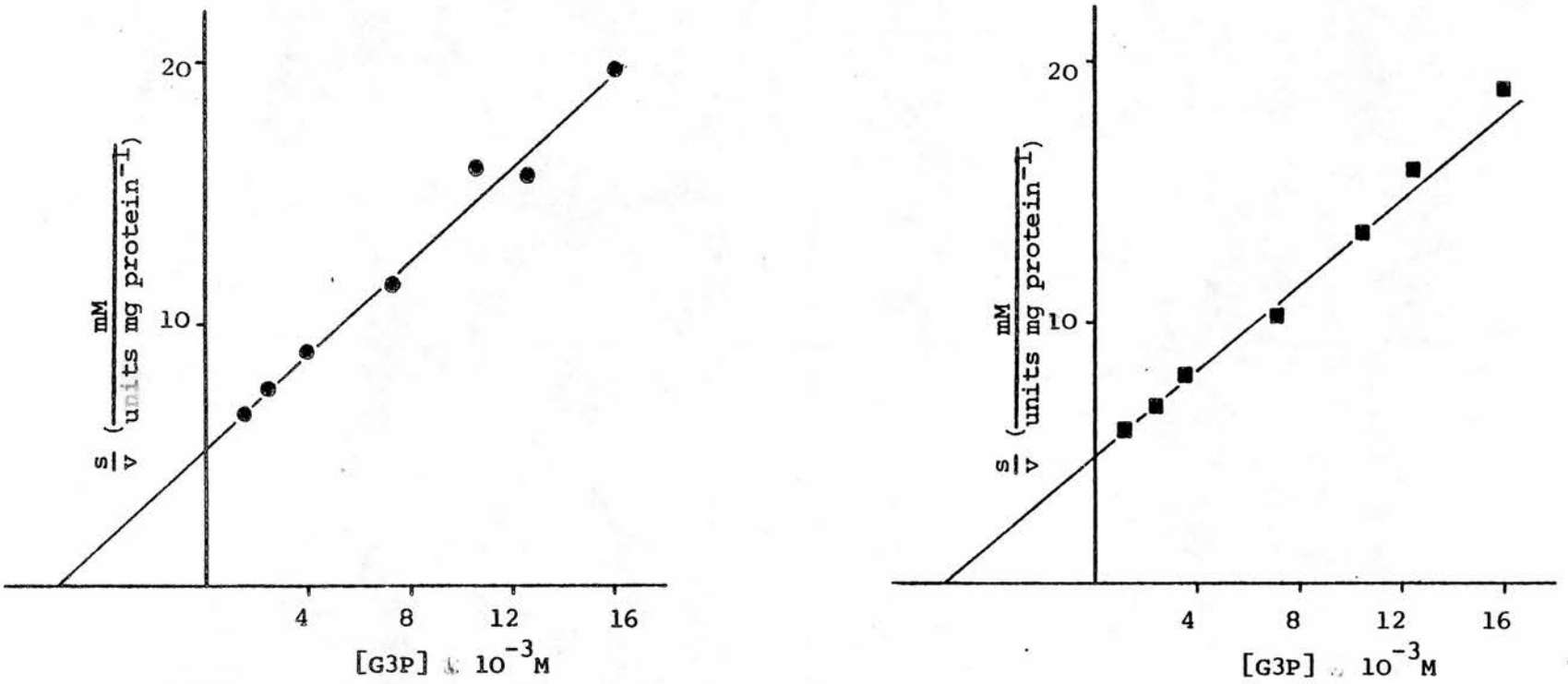
The initial velocities were measured immediately after the addition of the enzyme and the data plotted as a function of the ATP concentration. The results are shown in Figure 3.21. Apparently typical rectangular hyperbolae are observed with the enzyme from both parent and resistant lines. For the estimation of the Michaelis-Menten constants, the plot of s/v vs s (a Hanes plot) was used. Figure 3.22 shows the converted data to give

Figure 3.23: The effect of the G3P concentration on the activity of semipurified phosphoglycerate kinase from parent and resistant lines.




All the experimental conditions are described in Figure 3.21, with the difference that G3P is the variable substrate. The results are presented as the enzyme activity as a function of the G3P concentration (ATP constant at $3 \times 10^{-3}$ M). ●-● parent line; ■-■ resistant line.

Figure 3.24: The effect of ATP concentration on the activity of phosphoglycerate kinase: Hanes plot.




The analysis and presentation of the data are the same as in Figure 3.22. ●-● parent line; ■-■ resistant line.

linear biphasic plots. At approximately 1.5 x $10^{-3}$M ATP an inflection appeared in the plots from both parent and resistant enzyme. Biphasic double reciprocal plots have been previously reported for PGK (Yoshida and Watanabe, 1972; Schierbeck and Larsson-Raznikiewicz, 1979; Scopes, 1978a) when the initial velocities are studied as a function of the nucleotide concentration. It has been suggested by these authors that this is a characteristic of double binding sites for the nucleotide with different Km values. Only one active centre, however, is suggested for the enzyme (Schierbeck and Larsson-Raznikiewicz, 1979). In the present experiments the apparent Km values were 0.9 and 0.3 x $10^{-3}$M ATP, the values being very similar in both parent and resistant line. The kinetic parameters for the enzyme from both sources, with discussion, is presented in Section 3.5.5.

b. Effect of G3P concentration

The influence of G3P concentration on the initial velocity was investigated at a saturating concentration of ATP. The rest of the experimental conditions were exactly the same as for the estimation of the kinetic parameters of the cosubstrate ATP.

It is observed in Figure 3.23 that the initial velocities as a function of the concentration of G3P approach again rectangular hyperbolic for both parent and resistant line. After transformation of the data straight lines are observed (Figure 3.24). The information with respect to the number of binding sites for G3P, is rather more controversial than for ATP since Schierbeck and Larsson-Raznikiewicz (1979), have reported that the enzyme has two binding sites for the substrate. However, Scopes (1978b), from binding studies using a gel filtration technique, has suggested that there is only one binding site for G3P.

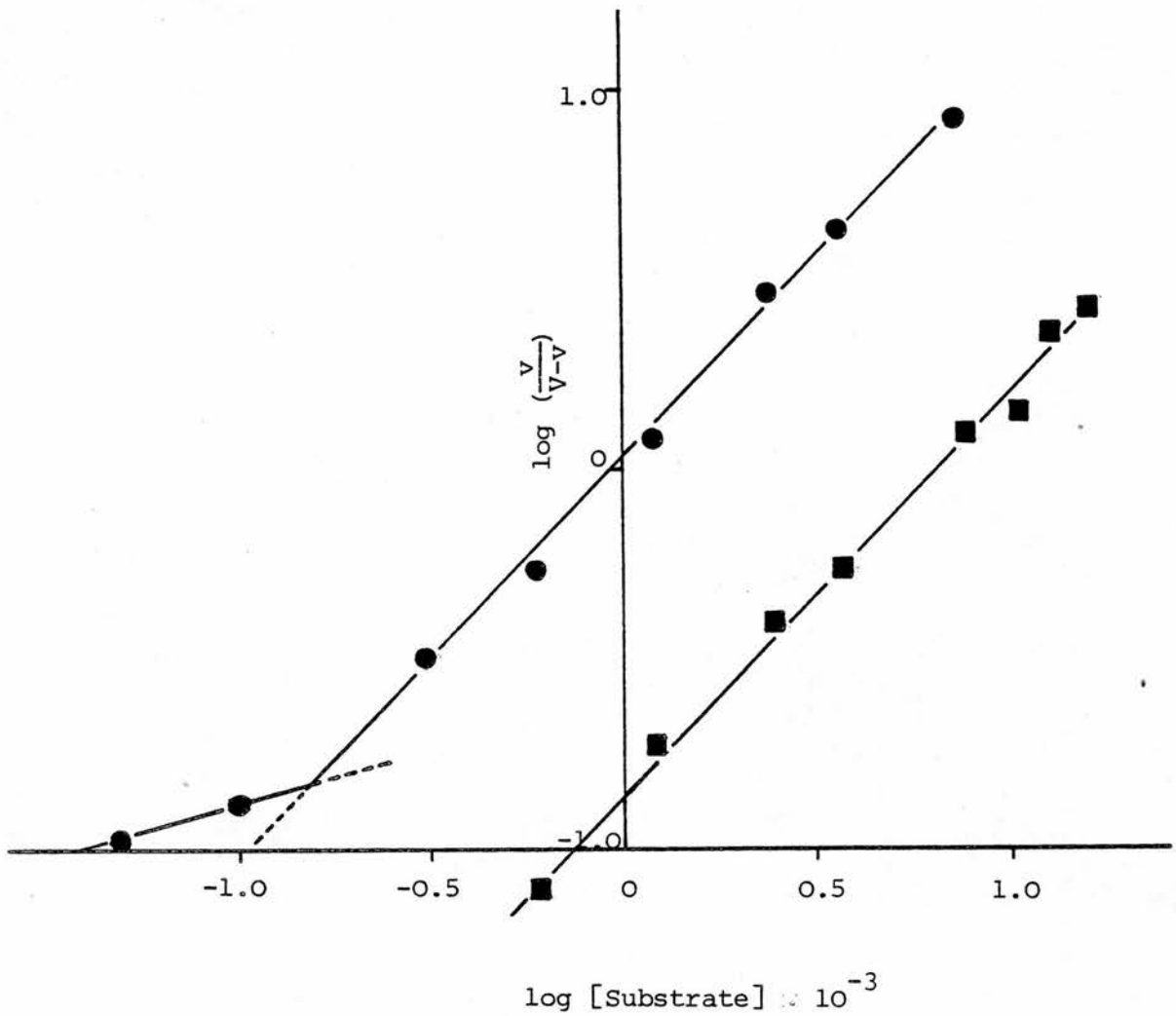
Another important feature of the enzyme is the already reported substrate activation when studying the back reaction (Scopes, 1978a;

Table 3.8: The kinetic parameters of phosphoglycerate kinase from parent and resistant lines.

| ATP | $K_{m_1}$ | $K_{m_2}$ (x $10^{-3}$ M) | $V_{max}$ (µmol $min^{-1}$ mg pro | |
|---|---|---|---|---|
| Parent | 0.89±0.22 | 0.32±0.09 | 0.90±0.18 | (3) |
| Resistant | 0.85±0.30 | 0.30±0.11 | 0.85±0.21 | (2) |
| G3P | | | | |
| Parent | 5.60±0.42 | | 1.10±0.28 | (3) |
| Resistant | 5.80±0.40 | | 1.15±0.32 | (2) |

The results presented in Figure 3.22 and 3.24 are presented as mean ± standard deviation of the mean; in parenthesis number of experiments.

Figure 3.25: The effect of ATP and G3P concentration on the activity of phosphoglycerate kinase: Hill plot.




The data from Figure 3.21 and 3.23 are presented using the transformat log $(\frac{v}{V_{max}-v})$ vs log [S]. Linear regression of the data was carried ou as described in Materials and Methods (Section 2.24). ●-● [ATP]. ■-■ [G3P].

Larsson-Raznikiewciz, 1967). This characteristic can be detected by studying any change in the absolute value of the Hill coefficient (Hill, 1910). Further analysis of the data was carried out by transforming the data using the Hill plot, the slope of the straight line obtained representing the n value.

The results of this analysis are presented in Figure 3.25. It was found that when ATP was the independant variable a biphasic plot emerged, with n = 0.28 at low concentrations of substrate and n = 1.08 at higher concentrations. The situation was not exactly the same for G3P which showed an apparently perfect straight line with an n value of 1.06. Therefore the data suggest that there was activation when the variable substrate was ATP. However when G3P was used there was either not activation or under the conditions of the assay the enzyme was fully activated as values lower than n = 1 were not observed.

c. Kinetic parameters of phosphoglycerate kinase

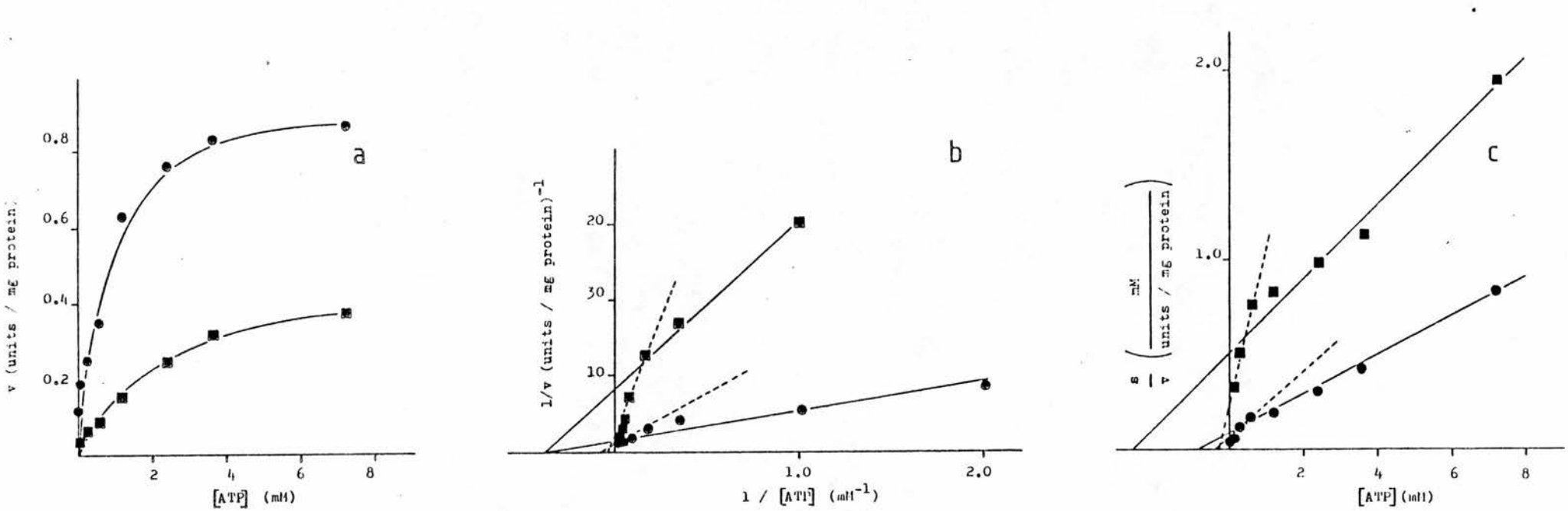
The values for the Michaelis-Menten constants are collected in Table 3.8. Apparently there are no significant differences occurring as a result of the development of resistance at the level of this particular enzyme.

The specific activities of the enzymes as judged from the $V_{max}$ are also the same. Using the same reasoning as for that used with PK, these conclusions do not preclude the possibility of changes in the sensitivities of the enzyme to melarsen oxide. The data corresponding to the inhibition of the two enzymes, from parent and resistant trypanosomes, is presented after a closer description of the mechanism of action of the enzyme in the next section.

3.5.4 Mechanism of action of phosphoglycerate kinase

It has been mentioned in the Introduction on PGK that the enzyme has a complex catalytic mechanism which is difficult to explain in simple

Figure 3.26: The effect of G3P on the binding of ATP in a semipurified phosphoglycerate kinase.




Aliquots of semipurified enzyme were preincubated with the components of the assay mixture containing a constant concentration of G3P. The mixture was incubated for 5 minutes at 25°C and the reaction started by addition of the variable substrate ATP. The initial rates were measured and Lineweaver-Burk and Hanes plots derived as described in Materials and Methods (Section 2.10 and 2.25). Reaction mixture with: ●-● $6 \times 10^{-3}$ M G3P and ■-■ $1 \times 10^{-3}$ M G3P.

terms using the models available for this purpose. However some of the evidence presented suggests a rapid equilibrium random mechanism in which the binding of one substrate does not affect the binding of the other (Larsson-Raznikiewicz and Ardivison, 1971; Lee and O'Sullivan, 1975).

An attempt to characterise the mechanism of action of the trypanosome enzyme was made in order to compare the parasite enzyme with that of the host.

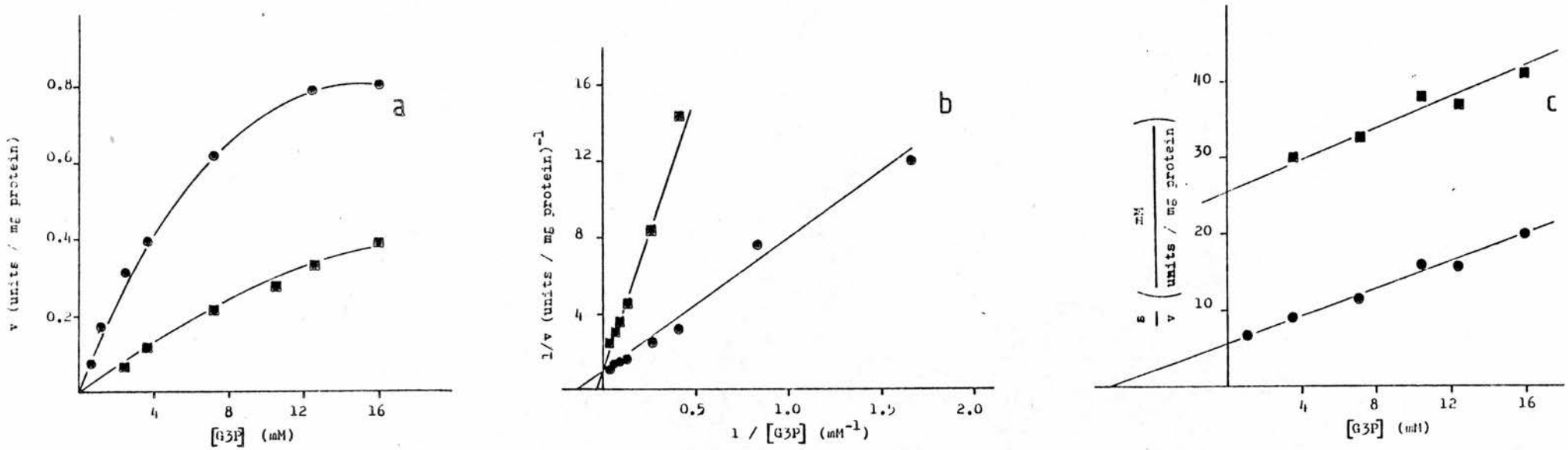
a. Effect of G3P on the binding of ATP

There are different steady-state kinetic methods available for elucidating the mechanism of addition of substrates and release of the products (Cornish-Bowden, 1979); one of these methods is the measurement of the initial velocities in the absence of products at different concentrations of the cosubstrate. The information gathered in this fashion gives some preliminary evidence on the type of mechanism involved.

Figure 3.26a shows the initial velocities for PGK as a function of the concentration of ATP at two fixed concentrations of G3P (1 and $6 \times 10^{-3}$M), the assay conditions are as described in Materials and Methods. By looking at the plot of v against s it can be seen that there is a considerable reduction in the $V_{max}$ at low concentration of the cosubstrate G3P. To obtain more information about the binding of the substrates, linearisation of the hyperbola is shown in Figure 3.26b. A very complex pattern is obtained, possibly due to the reported possibility of two binding sites. Two straight lines are apparently present which intersect at common points ($K_{m_1}$ $0.26 \times 10^{-3}$M and $K_{m_2}$ 1.25-$2.50 \times 10^{-3}$M).

The data treated in this fashion were difficult to interpret because of the concentration of the points at the high substrate range of the scale. However at least for $K_{m_1}$ there is no clear evidence of dependance on the concentration of G3P; $K_{m_2}$ was more difficult to interpret as it appeared with a competitive component which produces a

Figure 3.27: The effect of ATP on the binding of G3P in semipurified phosphoglycerate kinase.




All the experimental conditions were the same as Figure 3.26; concentration of ATP was kept constant and the reaction was started by addition of the variable substrate G3P. The initial rates were measured and the data were analysed as in the figure mentioned above. Reaction mixture with: ●-● $3 \times 10^{-3}$ M ATP and ■-■ $0.9 \times 10^{-3}$ M ATP.

higher apparent $K_m$.

In order to clarify the situation the data was transformed again with the help of another primary plot (Figure 3.26c). The complex pattern was observed at low substrate concentration and the independance of the $K_{m_1}$ on the concentration of G3P is substantiated. What originally looked like a competitive component is corroborated but with the difference that the inhibition is more of the mixed type. Different possibilities regarding the significance of this pattern of inhibition can be proposed; these are presented in the Discussion.

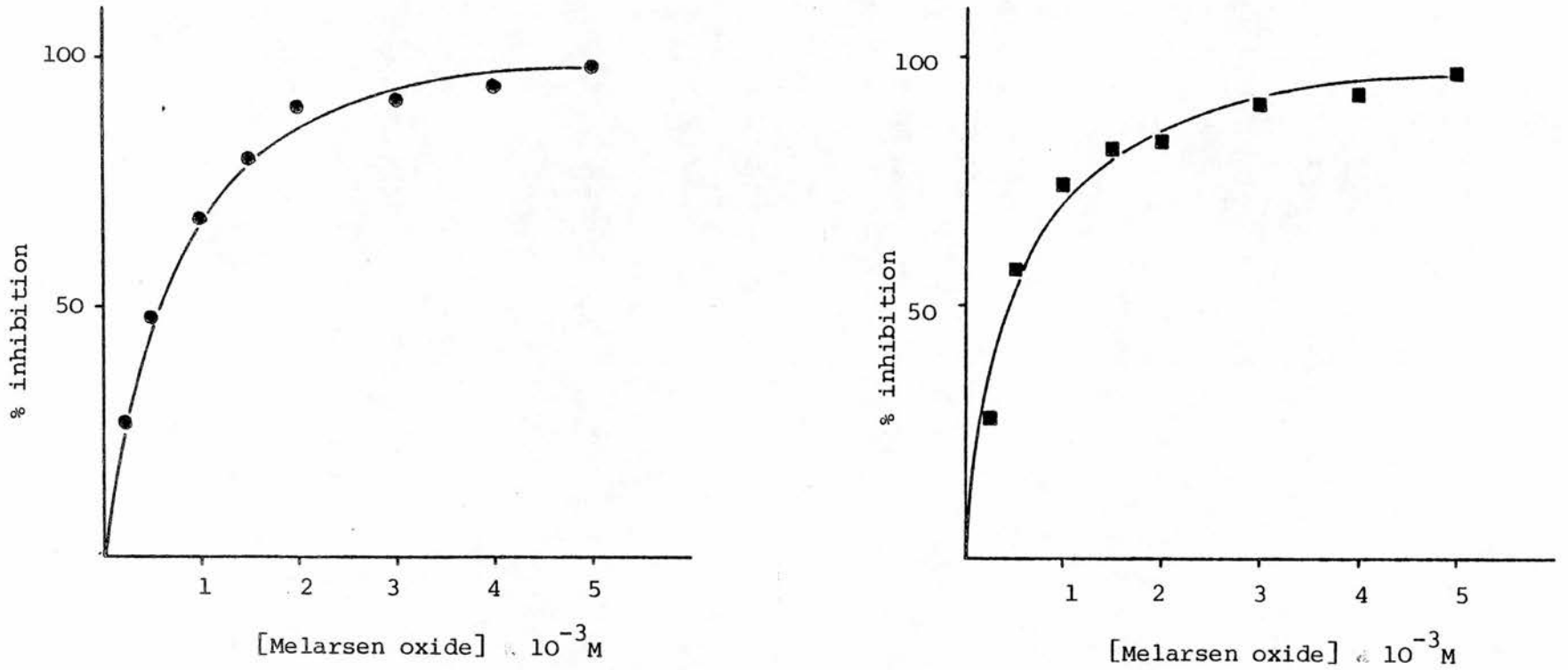
b. Effect of ATP on the binding of G3P

The effect of the other substrate (ATP) on the binding of G3P can be studied only by reversing the conditions of the assay. The results of the influence of the nucleotide on the initial velocities as a function of G3P concentration are shown in Figure 3.27a. The same transformations showed linear plots as seen in Figure 3.27b. The lines did not converge to the same point on the horizontal axis nor did they show the pattern for a substituted enzyme mechanism which in this particular plot appears as a set of parallel lines. The observation is further corroborated when the other transformation is carried out (Figure 3.27c). Apparently what is observed is a classical pattern of competitive inhibition when the concentration of ATP is varied.

3.5.5 The inhibition of phosphoglycerate kinase by melarsen oxide from parent and resistant lines

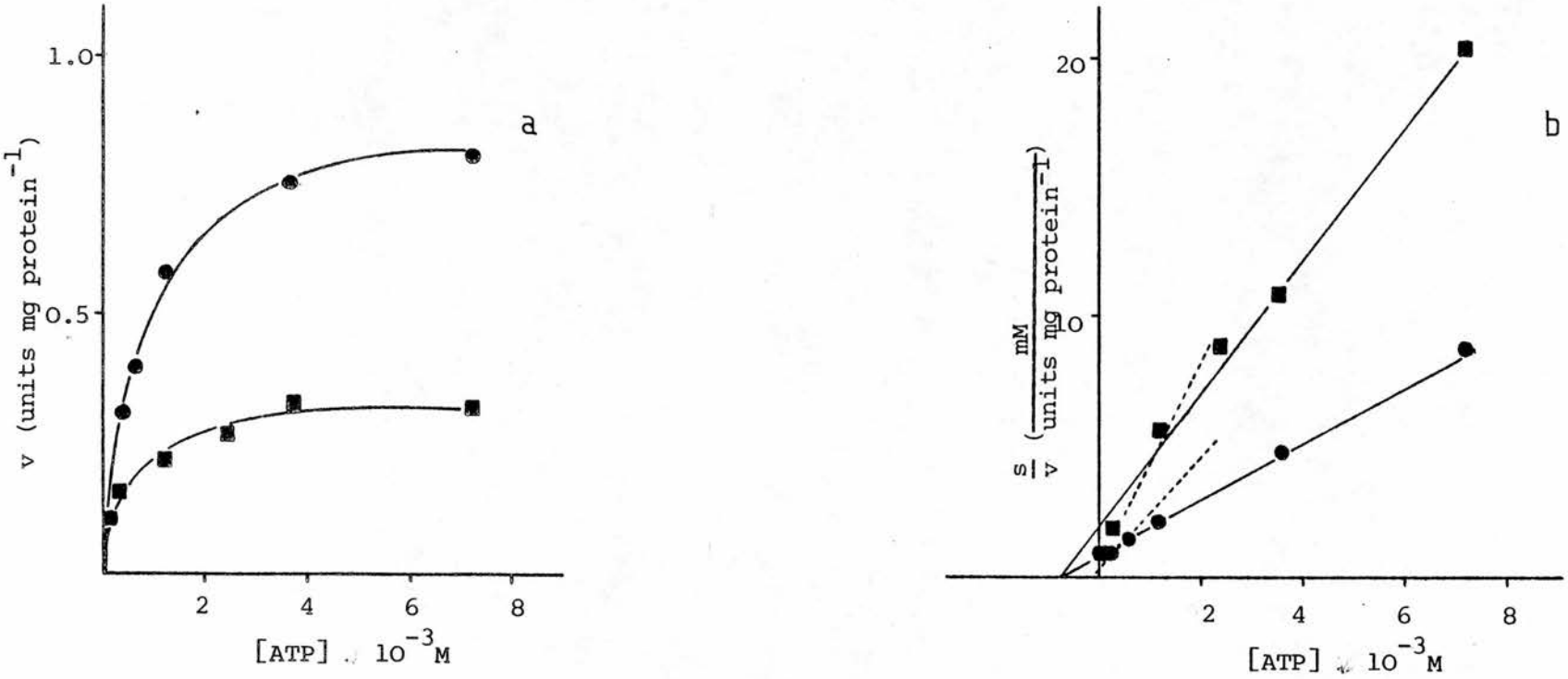
It was found that PGK from the parent and the resistant lines has the same kinetic parameters. The enzyme from parent and resistant line was prepared as reported at the beginning of this chapter and the standard assay constituents were used. The enzyme was incubated for 5 minutes in the presence of melarsen oxide and the reaction was started by the addition of the substrates (ATP and G3P). Addition of the substrates to the medium produced protection against inhibition.

Figure 3.28: The effect of melarsen oxide on the activity of phosphoglycerate kinase.




Enzyme samples were prepared and estimated as described under Materials and Methods (Sections 2.9 and 2.10.2). Aliquots of enzyme were preincubated for 5 minutes with different concentrations of melarsen oxide and the components of the assay mixture. The reaction was started by addition of ATP ($3 \times 10^{-3}$M) and G3P ($10 \times 10^{-3}$M). The results are presented as percentages of inhibition compared to the uninhibited enzyme as a function of drug concentration. ●-● parent line; ■-■ resistant line.

Figure 3.29: The effect of melarsen oxide on the kinetics of semipurified phosphoglycerate kinase with respect to ATP.




Aliquots of semipurified enzyme were preincubated with and without melarsen oxide (0.5 x $10^{-3}$M) for 5 minutes at 25°C with the components of the assay mixutre (G3P 10 x $10^{-3}$M). The reaction was started by addition of the variable substrate ATP. The initial velocities were measured and Hanes plots were derived as described under Materials and Methods (Section 2.25). ●-● without melarsen oxide; ■-■ with melarsen oxide.

The results in Figure 3.28 represent the residual activities compared to the control without drug, as a function of the melarsen oxide concentration. The patterns of inhibition were apparently similar for the enzymes from parent and resistant lines of T. brucei, the $I_{50}$ values being 0.60 and 0.46 x $10^{-3}$M respectively.

3.5.6 Mechanism of inhibition by melarsen oxide

It was found in the last section that melarsen oxide produced an inhibitory effect on the action of PGK. The aim of the next series of experiment was to characterise the mechanism of inhibition. The enzyme (parent line) was prepared and assayed using the standard conditions already described, the only modification being that the enzyme was incubated with the components of the assay system excepting the variable substrate to be investigated. The reaction was started after 5 minutes of incubation by addition of the variable substrate. The cosubstrate was kept constant at saturating concentration.

a. Effect of melarsen oxide on ATP binding

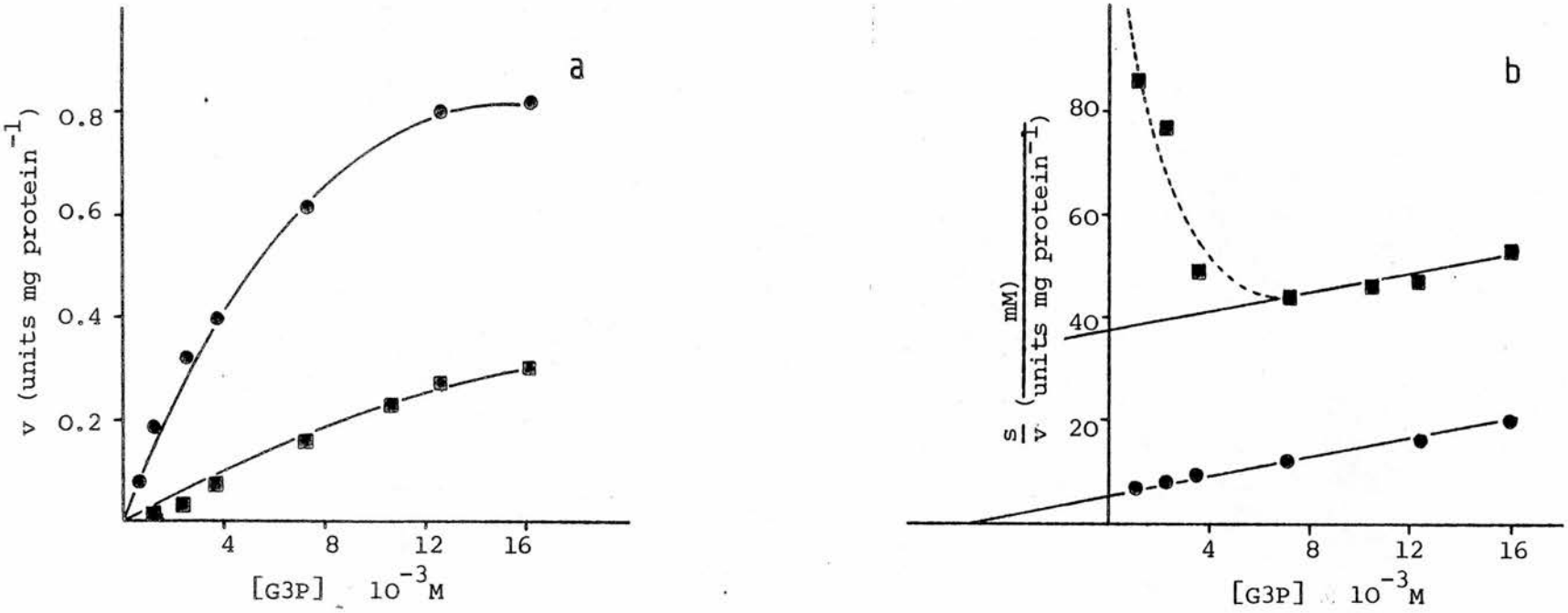
When the concentration of ATP was varied in the presence of 0.5 x $10^{-3}$M melarsen oxide and a fixed concentration of G3P (10 x $10^{-3}$M), it was observed that there was a considerable reduction in the activity of the enzyme as seen in Figure 3.29a.

A further analysis of the data with the linear transformation (s/v vs s) suggested that the binding of ATP is independant of the drug (Figure 3.29b). The maximal activity of the reaction was decreased from ca. 0.85 µmol $min^{-1}$ $mg\ protein^{-1}$ to 0.35 µmol $min^{-1}$ $mg\ protein^{-1}$. Thus it is suggested that the pattern of inhibition observed is apparently one of non-competitive inhibition as melarsen oxide (at 0.5 x $10^{-3}$M) does not interfer with the binding of ATP but with the catalytic activity.

b. Effect of melarsen oxide on G3P binding

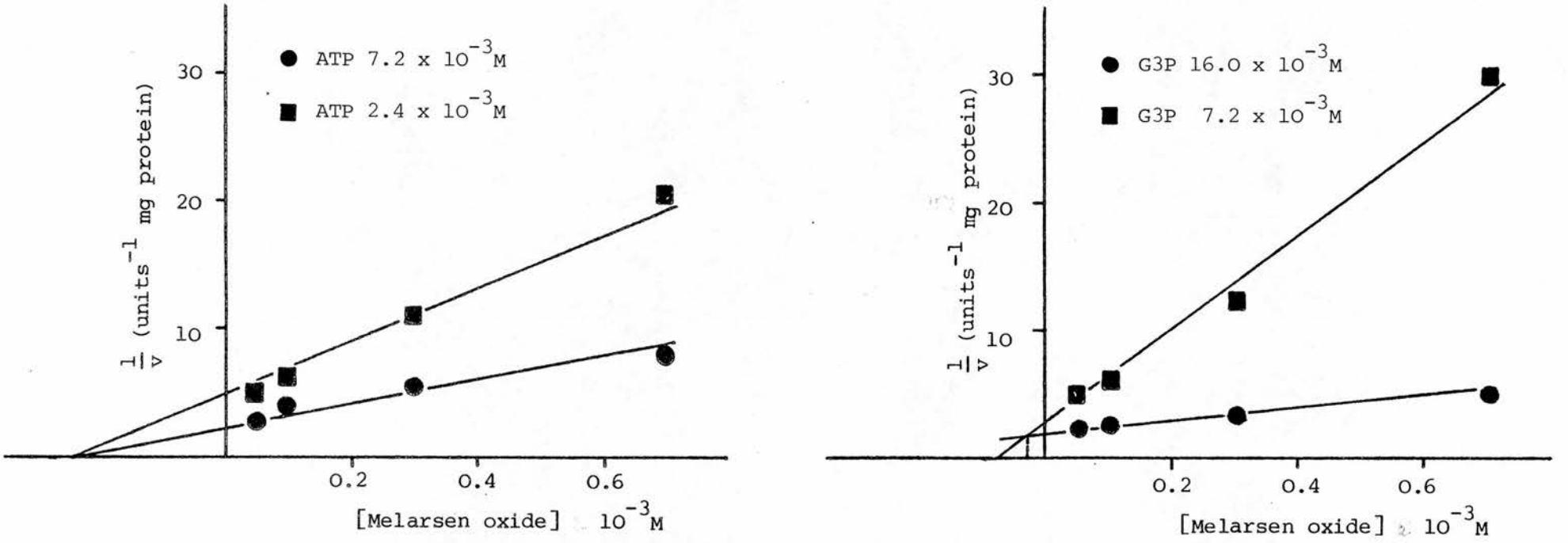
The effect of melarsen oxide on the binding of G3P was studied in

Figure 3.30: The effect of melarsen oxide on the kinetics of semipurified phosphoglycerate kinase with respect to G3P.




All the experimental conditions were as described in Figure 3.29 (ATP 3 x $10^{-3}$M). The reaction was started by the addition of the variable substrate G3P. The initial velocities measured and Hanes plots were derived as described under Materials and Methods (Section 2.25). ●-● without melarsen oxide; ■-■ with melarsen oxide.

Figure 3.31. The estimation of the inhibitory constant ($K_i$) for ATP and G3P in semipurified phosphoglycerate kinase.




Enzyme samples were prepared and estimated as described under Materials and Methods (Sections 2.9 and 2.10.2). For the estimation of the $K_i$ value with respect to ATP, aliquots of enzyme were incubated for 5 minutes with the components of the assay mixture at different concentrations of melarsen oxide. G3P concentration was constant at 10 x 10$^{-3}$M. The reaction was started in two series of cuvettes by addition of ATP (2.4 x 10$^{-3}$ and 72 x 10$^{-3}$M respectively). For the estimation of the $K_i$ value with respect to G3P the same procedure was used, ATP concentration being kept constant (3 x 10$^{-3}$M). The reaction was started by addition of G3P, 7.2 x 10$^{-3}$M and 16 x 10$^{-3}$M respectively. The initial rates were measured and Dixon plots derived as described in Section 2.25.

a similar fashion. This time the concentration of the nucleotide was kept constant at $3 \times 10^{-3}$M.

The pattern of inhibition found was typical of competitive inhibition at above $7 \times 10^{-3}$M G3P as seen in Figure 3.30a,b. It may be seen in the linear transformation of the data that some points departed from linearity at low concentrations of G3P.

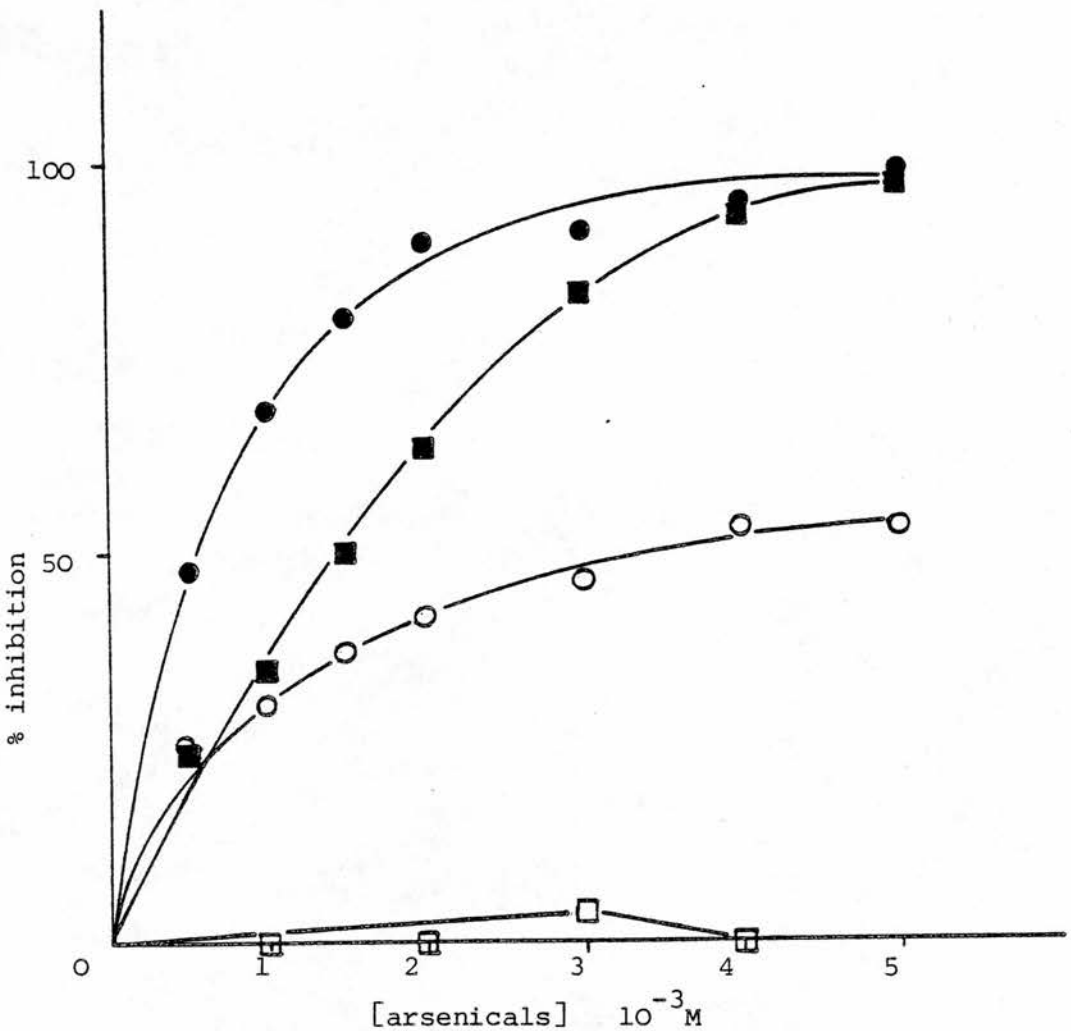
As mentioned above the inhibition produced by melarsen oxide on the binding of G3P affected mostly the binding of the substrate producing an increase in the apparent Km.

The overall inhibition presented is rather similar to the one reported for PK for the same drug (Flynn, 1971); it was found that PK was inhibited noncompetitively with respect to the nucleotide (ADP), and competitively for the cosubstrate (PEP).

3.5.7 The inhibitory constant for phosphoglycerate kinase

The method of Dixon (1953) was used to estimate the inhibitory constants for both substrates of phosphoglycerate kinase. The results were obtained using the standard assay system for the enzyme with the difference that the system was incubated with different concentrations of drug for 5 minutes. The reaction was started by the addition of the variable substrate, the other cosubstrate already being present at a constant concentration in the assay system. The data were presented as the mean of duplicates for either substrate as a function of the concentration of melarsen oxide in Figure 3.31. The results obtained apparently substantiated the information already discussed, non-competitive inhibition is found with ATP and competitive inhibition with G3P. The values of the inhibitory constants were $K_i = 0.25 \times 10^{-3}$M and $0.03 \times 10^{-3}$M melarsen oxide for ATP and G3P respectively.

Figure 3.32: The effect of different arsenicals on the activity of phosphoglycerate kinase.




Aliquots of semipurified phosphoglycerate kinase were incubated for 5 minutes with different concentrations of organic arsenicals and the components of the assay mixture. The reaction was started by addition of ATP ($3 \times 10^{-3}$ M) and G3P ($10 \times 10^{-3}$ M). The results are presented as percentages of inhibition compared to the uninhibited enzyme. ●-● melarsen oxide; ■-■ phenyl arsenoxide; O-O reduced atoxyl; □-□ arsenite.

3.5.8 The effect of different arsenicals on phosphoglycerate kinase

The effects of the inhibition of PGK by other arsenicals were shown in Figure 3.32; the experimental conditions were similar to those used when studying the inhibition of melarsen oxide. Significant differences are found with regard to the sensitivity to the different structures tested. The $I_{50}$ values presented are assumed to represent the overall effectiveness of the drugs to produce inhibition of PGK.

The order of effectiveness was melarsen oxide>phenylarsenoxide> p-aminophenylarsenoxide with $I_{50}$ values of 0.55; 1.45; and 3.40 x $10^{-3}$M respectively. Inorganic arsenite did not show inhibition within the range of concentrations used. Neither melamine nor the pentavalent arsenical sodium melarsen inhibited the enzyme at a concentration of 5 x $10^{-3}$M.

As described in Material and Methods the standard assay for PGK uses glyceraldehyde phosphate dehydrogenase as a coupling system with the subsequent oxidation of NADH. It was found that the coupling enzyme was not sensitive to most of the arsenicals used when the reaction was started, after incubation with the drug, by addition of excess of yeast PGK. An exception should be made for p-aminophenyl-arsenoxide which produced 30% inhibition at the highest concentration of drug used, therefore it is suggested that the $I_{50}$ value for this drug is lower than the one presented above.

3.5.9 Summary

As mentioned in the Introduction the possibility of enzymic changes resulting in the development of resistance to active drugs has been suggested before. However little attention has been paid to this particular aspect of the development of resistance to arsenical drugs. The experiments designed with PK and PGK from parent and resistant lines were performed with the objective of gaining some insight into:

1) whether or not an increased specific activity of either of these enzymes was present in order to overcome the action of the drug, and 2) whether modification of the kinetic parameters or sensitivity to the drug has occurred in the resistant line. Neither possibility is substantiated as the two enzymes tested were not different in parent and resistant lines.

The results obtained however corroborate the hypothesis postulated in the metabolic studies, that a permeability barrier for the drug exists in the resistant line. As the possibility of modification of the target enzymes was discarded, the alternative of differential uptake of the drug in the resistant line remains to be confirmed experimentally.

With regard to the data obtained with PK, the results corroborate those reported by other authors (Flynn and Bowman, 1980). It was found that the parent enzyme has a sigmoidal response with respect to PEP with $S_{50}$ values = $1.60 \pm 0.30 \times 10^{-3}$M and a Hill coefficient = $2.16 \pm 0.30$. The enzyme also has a typical Michaelis-Menten pattern with respect to ADP with $K_m$ value = $3.17 \pm 0.22 \times 10^{-3}$M.

PGK received considerable attention. It was found that the enzyme has a hyperbolic response of velocity as a function of G3P and ATP concentrations. However upon transformation of the data a linear biphasic response was obtained for ATP with $K_{m_1}$ value = $0.90 \times 10^{-3}$M and $K_{m_2}$ = $0.30 \times 10^{-3}$M. The data is interpreted as the enzyme having two binding sites for the substrate but with different affinities. Transformation of the data for G3P produced simple linear responses, evidence of a single binding site with a $K_m$ value = $6.0 \times 10^{-3}$M (approximately). The experiments on the mechanism of action of the enzyme showed a very complex picture; some evidence points towards a sequential mechanism of action.

Finally PGK was inhibited by melarsen oxide, competitively with respect to G3P and noncompetitively with respect to ATP. The $K_i$ values were 0.03 x $10^{-3}$M and 0.25 x $10^{-3}$M melarsen oxide respectively. Other aromatic arsenicals also inhibit the enzyme.

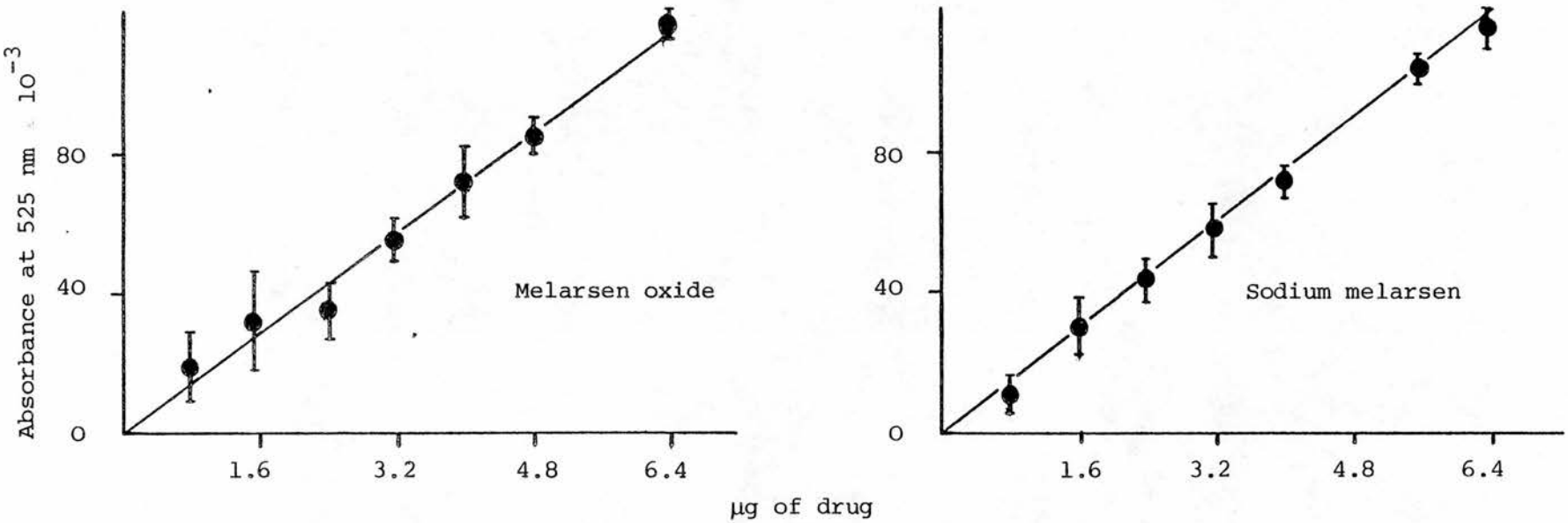
## 3.6 Drug transport studies

In the last two chapters of this thesis, an attempt has been made to rationalise the problem of drug resistance in the newly developed melarsen oxide resistant line. The possibility of a metabolic alteration resulting in the development of the new character was considered and although it was found that the resistant line showed some minor metabolic differences, this could not explain directly the development of the new character. However, the metabolite inhibition studies suggested the possibility of a permeability barrier for the drug in the resistant line.

The hypothesis suggested above does not eliminate the alternative of modified drug targets to account for the phenomenon of drug resistance. Therefore a closer look was taken, first at pyruvate kinase, a demonstrated target for melarsen oxide and secondly at phosphoglycerate kinase, another enzyme potentially involved in the development of the phenomenon because of its sensitivity to melarsen oxide. The kinetic parameters and the inhibition patterns of both enzymes did not show any significant differences to explain the development of the resistant character.

The alternative of differences in the mechanism of uptake of the drug can be singled out as the most viable proposition from the experiments performed up to this point. Indeed this alternative has been suggested before to explain the phenomenon of resistance to organic arsenicals (Yorke and Murgatroyd, 1930; Eagle and Magnuson, 1944; Williamson and Rollo, 1959). However, most of the studies done so far have only taken into account the abilities of the parent and resistant parasites to accumulate the drugs over relatively long periods of time. Therefore the main objective of this section was to characterise the mechanism of uptake of melarsen oxide, in kinetic terms, in both parent and resistant lines of *T. brucei*.

Figure 3.33: Estimation of melarsen oxide and sodium melarsen using the silver diethyldithiocarbamate method.




Aliquots of standard drug solutions were placed in 125 ml conical flasks, and the organic digestion, arsine generation and subsequent trapping in AgDDC solution was carried out as described in Materials and Methods (Section 2.19). The absorbance was read at 525 nm in the AgDDC solution against appropriate blanks prepared using the same experimental conditions.

3.6.1 Estimation of the arsenical drugs using the silver diethyldithiocarbamate method

One of the first requirements for this kind of work is to have a suitable and sensitive assay system for the estimation of the drug being used. As discussed in the Introduction different methods are available. The method of choice should be accurate, reproducible and sensitive at low levels of the drug.

As described in Materials and Methods the system chosen was a colorimetric one, which uses silver diethyldithiocarbamate (AgDDC) as a chelating agent for inorganic trivalent arsenic. Upon formation of the complex with the metal a red colour is developed in the AgDDC solution in pyridine, which absorbs light at 525 nm.

Calibration curves were produced with melarsen oxide and sodium melarsen after digestion of the organic matrix, generation of arsine and trapping by AgDDC in solution. The results are presented in Figure 3.33 where the colour developed was measured in a triplicate set of standards for each drug. It was found that the standard curves were linear up to 6.5 µg of either drug. In general the standard deviations were higher towards the low drug concentrations but the linearity of the system was conserved. Standards were prepared with every set of unknowns because of the small day-to-day variation found with the calibration curves. It is concluded also that the sensitivity of the assay system is independant of the original state of oxidation of the arsenical moiety in the drug as there was a comparable level of sensitivity in both estimations.

3.6.2 Trypanosome motility test in the presence of melaminyl arsenicals

As described in Materials and Methods, an oxygen electrode chamber was used to incubate the trypanosomes at the temperature at which exposure to the drug is to be carried out. After addition of the drug it was

Table 3.9: The action of melarsen oxide on the motility of the parent line of T. brucei.

| [Melarsen oxide] x $10^{-6}$ | Time (seconds) 30 | 60 | 90 | 120 | 180 | 210 |
|---|---|---|---|---|---|---|
| 0 | +++ | +++ | +++ | +++ | +++ | +++ |
| 10 | +++ | +++ | +++ | +++ | +++ | ++ |
| 25 | +++ | +++ | +++ | +++ | +++ | ++ |
| 50 | +++ | +++ | +++ | +++ | ++ | + |
| 60 | +++ | +++ | +++ | ++ | + | +o |
| 70 | +++ | +++ | ++ | + | +o | +o |
| 80 | +++ | ++ | + | +o | +o | +o |
| 100 | +++ | ++ | +o | +o | +o | +o |

Trypanosomes from parent line were isolated from blood elements as described in Section 2.5, resuspended in PSGA, pH 8.0 and incubate in an oxygen electrode chamber at 21°C as described for uptake studie (Section 2.16) with different concentrations of melarsen oxide. Every 30 seconds approximately, the motility was monitored under phase contrast microscopy (400 x) and the population of trypanosomes evaluated as described under Materials and Methods (Section 2.7).

necessary to check that under the conditions of incubation the trypanosomes remained motile for enough time to ensure that uptake studies are a real feature of the population of living cells.

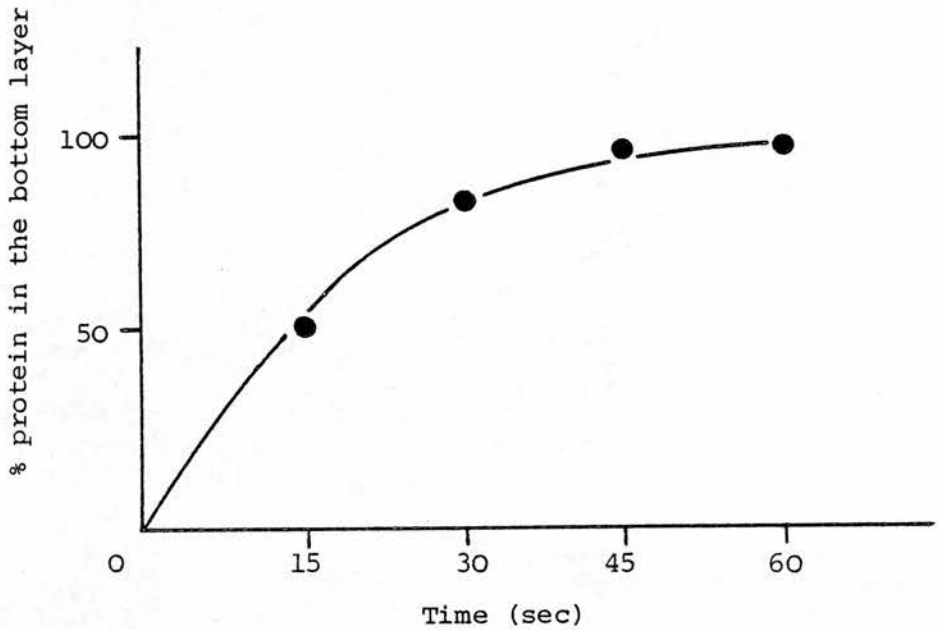
The concentration of trypanosomes used in these experiments was approximately equivalent to 5.0 mg of trypanosome protein/ml buffer (c.a. $10^8$ cells/ml). This high concentration of trypanosomes was chosen to increase the total concentration of drug taken by the parasites and to produce significant results with the drug assay system described in the last section. The first preliminary experiment was done at 21°C, the reason being that at higher temperatures the cells may metabolise the substrate available in a very short period of time.

After incubation of trypanosomes from both lines of parasites at different concentrations of melarsen oxide, the results shown in Table 3.9 were found. As observed in earlier motility test for the parent line the activity decreased more rapidly as the concentration of drug increased. It should be noticed that no apparent decrease in the motility of the resistant line was observed. However the main feature of this motility test is that up to 60 second incubation with up to 70 to 80 x $10^{-6}$M melarsen oxide the organisms retained full motility. This time was therefore chosen as the standard time of incubation for the uptake experiments with the concentrations of melarsen oxide mentioned above.

It is also suggested, that to produce a rapid effect like the one produced by melarsen oxide on sensitive cells, the mechanism of transport of the drug should allow the rapid translocation of the drug from the extracellular medium to the site of lethal effects.

Finally the motility was tested with sodium melarsen under the same conditions described for melarsen oxide and no effect was observed on this parameter in either parent or resistant lines even after an incubation period of 210 seconds.

Figure 3.34: Trypanosome protein clearance from the exposing solution using the silicone layer filtration technique.




Trypanosomes isolated as described in Section 2.5 were resuspended in PSGA buffer pH 8.0 to a final concentration of about 5 mg of trypanosome protein per ml suspension. Aliquots (0.5 ml) of this suspension were poured on to silicone sandwiches and centrifuged at 10,000 g for different time intervals. The results are presented as the percentage of protein in the bottom layer as a function of time.

3.6.3 Trypanosome protein clearance using the silicone layer filtration technique

A relatively rapid exposure technique should be used in order to have a reliable estimate of the initial rates of absorption of the drug (Halestrap and McGivan, 1979). The silicone layer filtration technique as described by Damper and Patton (1976b) was used for the uptake experiments in this thesis. The method was slightly modified to allow for the amount of trypanosomes required in the experiments, by increasing the volume of the microcentrifuge tube and doubling the volume of the incubation and the silicone layers. Further details of the technique are presented in Materials and Methods.

A preliminary experiment was performed to illustrate the clearance of trypanosome protein from the exposing solution, when submitted to centrifugation in a bench microcentrifuge at 10,000 g value for different periods of time. For this experiment the bottom layer of perchloric acid was substituted with PS buffer. The protein pellets formed after centrifugation were washed twice in saline and the protein estimated. The results of this experiment are shown in Figure 3.34. It was found that after centrifugation for 45 seconds practically all the protein had crossed the silicone layer and formed a pellet at the bottom of the tube. The standard time of 60 seconds for centrifugation was therefore chosen for experiments on the uptake of arsenical drugs.

3.6.4 Extracellular volume determination

One minor disadvantage of the silicone layer filtration technique is that when the parasites are centrifuged through the silicone layer, they drag some extracellular fluid. In the case of the uptake experiments this fluid will contain some free drug which is not bound to the parasite. In order to correct for this extracellular drug, a non-permeable marker was used to estimate the amount of extracellular fluid involved.

Table 3.10: Determination of extracellular volume carried through using the silicone layer filtration technique.

| cpm in perchloric soluble layer | extracellular fluid (µl/2.5 mg trypanosome protein) |
|---|---|
| 252 | 12.64 |
| 254 | 12.72 |
| 261 | 13.09 |
| 261 | 13.04 |
| 226 | 11.33 |
| $\bar{x}$ | $12.56 \pm 0.72$ mean ± st.error of the mean |

All the experimental conditions regarding the purification of trypanosomes and incubation for uptake studies were the same as in Table 3.9. 10 µl of $^{14}C$ inulin (30,000 cpm approx.) were added to 2 ml of trypanosome suspension and incubate for 60 seconds or described in text. Aliquots (0.5M) were centrifuged using the silicone layer filtration technique and the radioactivity estimated in the perchloric acid soluble layer as described under Materials and Methods (Section 2.18). (See text.)

$^{14}C$ inulin has been used previously for this purpose with trypanosomes and found not to penetrate the parasites (Damper and Patton, 1976b).

To ensure that the polymer was free of low molecular weight components that may be taken up by the trypanosomes, the original source of inulin was purified using a small column of Sephadex G25. Details of purification are found in Materials and Methods.

Table 3.10 shows the results of a representative experiment in which $^{14}C$ inulin (3000 cpm in 10 µl) was added to 1.5 ml of trypanosome suspension. After centrifugation, samples from the perchloric acid soluble layer were estimated by scintillation counting in a water soluble scintillation fluid (see Materials and Methods). The results obtained after correcting for acid quenching were consistant in the different samples taken; the mean value being $12.56 \pm 0.72$ µl of extracellular fluid carried through by 0.5 ml of a trypanosomal suspension of approximately 5.0 mg/ml (parent line).

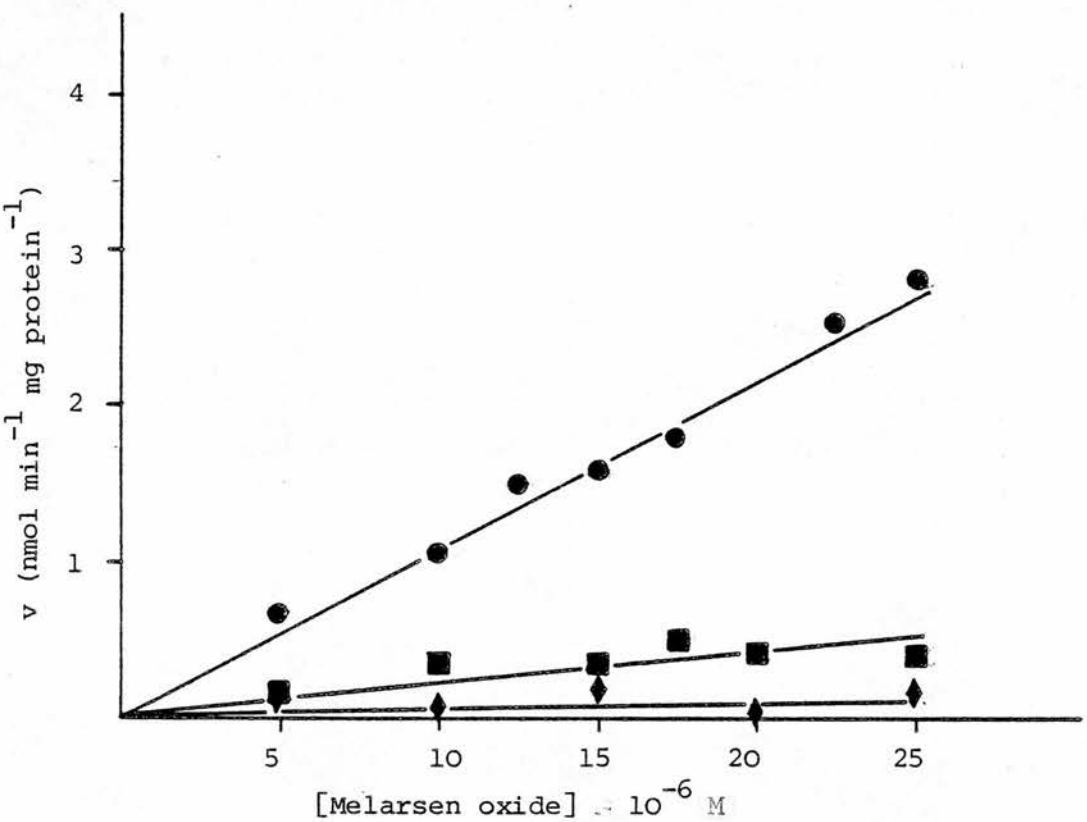
The value obtained was constant even if the trypanosomes remained in contact with the inulin for different lengths of time of up to 4 minutes. It should be pointed out that the value obtained for the extracellular fluid volume is used to correct for the extracellular drug in all the uptake experiments in this thesis.

3.6.5 Distribution of melarsen oxide after centrifugation using the silicone layer filtration technique

After incubation of the parasites (parent line) in the oxygen electrode chamber using the standard conditions described in Materials and Methods, at a temperature of $21^{\circ}C$, an attempt was made to determine the distribution of the drug in the different layer of the silicone sandwich after centrifugation.

Upon contact with the perchloric acid layer the trypanosome protein denatures and two fractions appear; an acid soluble and an acid

Figure 3.35: The distribution of melarsen oxide after centrifugatio using the silicone layer filtration technique.




Trypanosomes from parent line were isolated from blood elements as described in Section 2.5, resuspended in BSGA pH 8.0, and incubated in an oxygen electrode chamber at 21°C with different concentrations of melarsen oxide. Aliquots were centrifuged after 60 seconds incuba using the silicone layer filtration technique and the drug estimated in the different layers of the centrifuge tube. The data were corrected for the extracellular drug component. Details of the methods are as described under Materials and Methods (Sections 2.15, 2.16, 2.17 and 2.19). ■-■ upper layer; ♦-♦ perchloric acid soluble layer; ●-● perchloric acid insoluble layer.

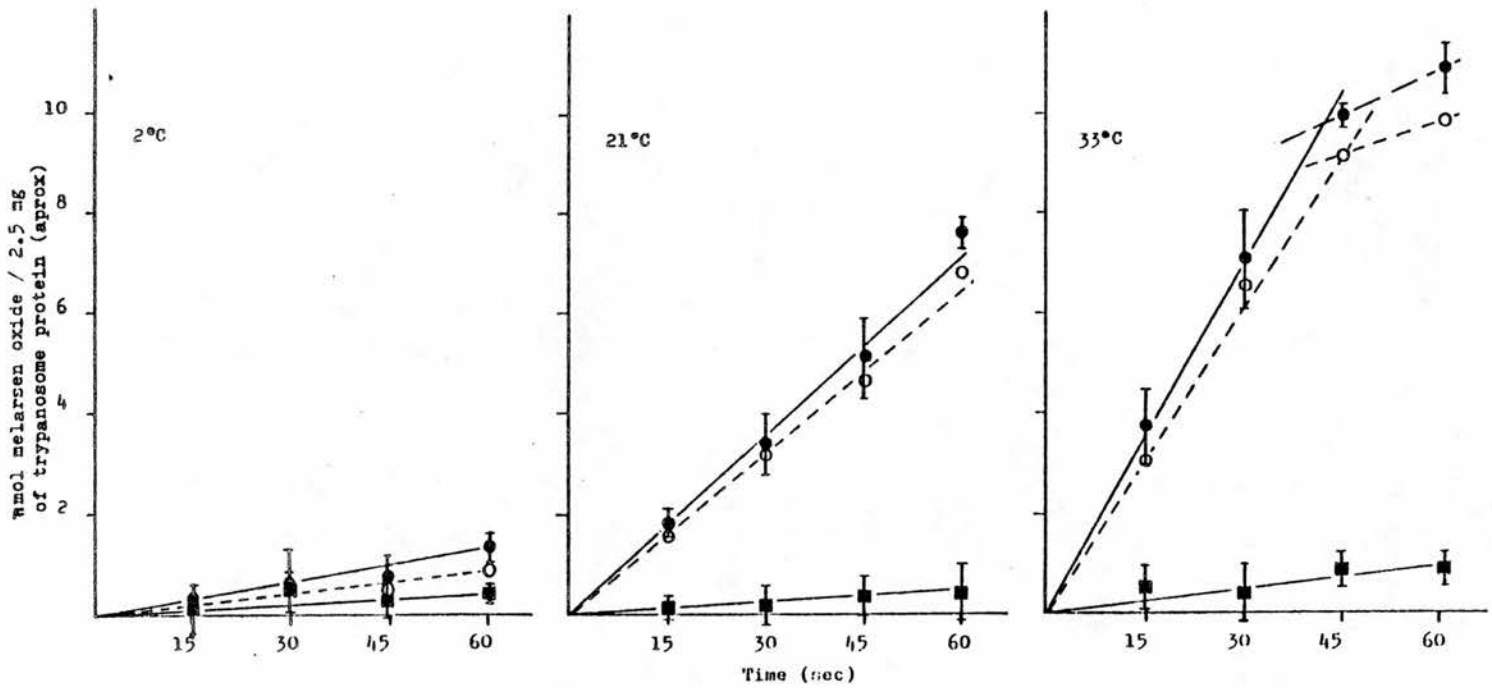
insoluble layer. For the purposes of the uptake studies it was necessary to know the distribution of the drug among the different fractions obtained. Samples were taken from the exposing solution (upper layer) and from the acid soluble and insoluble layer, after incubating the trypanosomes in different concentrations of melarsen oxide. The results obtained are shown in Figure 3.35. Itwas found that most of the drug was concentrated in the pellet (acid insoluble layer). It should be noticed that the figures obtained for the concentration of the drug appearing in the acid soluble layer approached the expected theoretical value calculated using the figure obtained, in the last section, for the extracellular fluid drug carried through with the trypanosomes.

3.6.6 Time course of melarsen oxide uptake at different temperatures in parent and resistant lines

An evaluation of the mechanism of transport of the arsenicals should take into consideration the influence of the temperature on the rate of the transport as it has been found that different transport systems studied show different responses with respect to this parameter (Goldman, 1973).

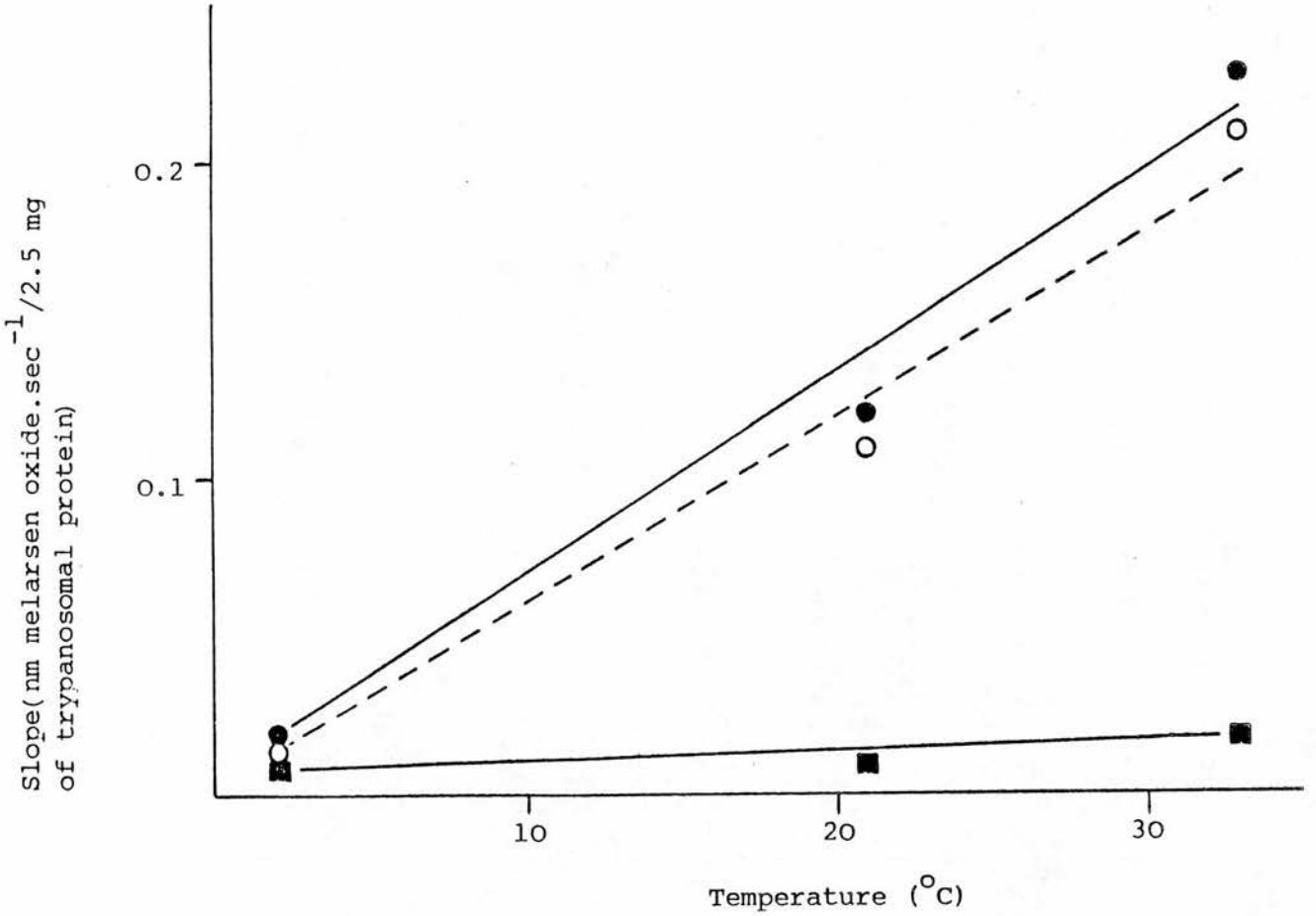
Therefore an experiment to illustrate the effect of temperature on the rate of transport, in both parent and resistant lines was performed. As reported in Materials and Methods the incubation of the trypanosome suspension, from both sources, was carried out in an oxygen electrode chamber coupled to a variable temperature water bath. The suspensions of trypanosomes were added to the chamber and allowed to equilibrate to the required working temperature (0.5 min for $2^{o}C$, 3 min for $21^{o}C$ and 7 min for $33^{o}C$). As soon as the required temperature was reached, melarsen oxide was added to the suspension to a final concentration of $80 \times 10^{-6}M$. Samples were taken from the oxygen electrode chamber every 15 seconds, loaded onto the top of a silicone sandwich

Figure 3.36: The effect of the temperature on the uptake of melarsen oxide in parent and resistant lines.




Trypanosomes from parent and resistant lines were isolated from blood elements as described in Section 2.25 and resuspended in PSGA buffer pH 8.0. The suspensions were incubated with 80 x $10^{-6}$ M melarsen oxide at different temperatures and at every 15 seconds; aliquots (0.5 ml) were centrifuged using the silicon layer filtration technique. The drug was estimated in the perchloric acid layer. The results are presented as the drug concentration found in the layer as a function of time for parent and resistant lines respectively. The data were corrected for the extracellular drug component. Details of the methods are as described under Materials and Methods (Sections 2.15, 2.16, 2.17 and 2.19) and in the text. ●-● parent line; ■-■ resistant line; O-O parent line corrected for a diffusion component.

Figure 3.37: The effect of temperature on the rates of uptake of melarsen oxide in parent and resistant lines.




From the results obtained in Figure 3.6, the rates of transport for parent and resistant lines were calculated and the results are presented as a function of the temperature used. ●-● parent line; ■-■ resistant line; O-O parent line corrected for a diffusion component.

and centrifuged. Estimation of the drug was performed as reported in Materials and Methods. The results of a triplicate experiment are presented in Figure 3.36 where the concentration of drug found in the perchloric acid layer (soluble and insoluble) is shown as a function of time.

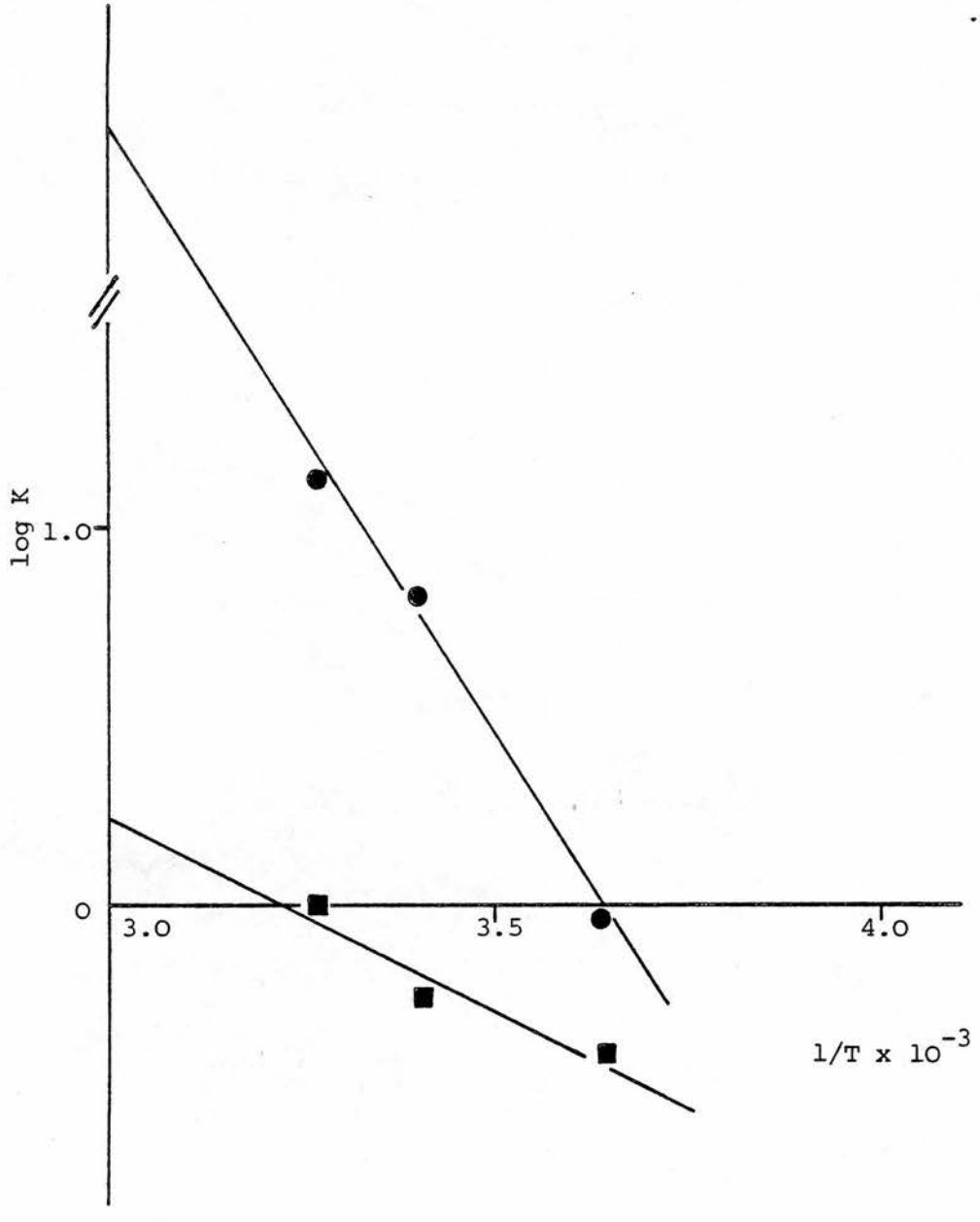
From these results it is observed in the first place that different patterns of drug uptake are present in the parent and the resistant lines, the latter showing a considerably lower rate of uptake; secondly that the rate of uptake in the parent line is affected considerably by the temperature.

To illustrate the effect of temperature on the rates of uptake in both parent and resistant lines, the slopes obtained from the regression lines were plotted as a function of temperature in Figure 3.37. It was found that the rate of transport was affected considerably in the parent line with a $Q_{10}$ of approximately 2.0. The situation was different with the resistant line, the $Q_{10}$ for the latter being 1.2.

Some alternatives have been suggested to explain stringent changes in the rate of uptake of a drug as a function of temperature. The first is that the drug is transported by a carrier which is energy dependant, hence the energy yielding process is disrupted by the decrease in temperature (Neame and Richards, 1972; Oxender, 1972). Another possible alternative is in terms of a change in the conformation of the membrane phospholipids, with a subsequent reduction in the fluidity of the membrane as the temperature is reduced (Kaback, 1968). The alternatives will be covered in more details in the Discussion.

With regard to the resistant line, it is possible that the temperature dependant transport may have disappeared and instead a low $Q_{10}$ (i.e. a simple diffusion) uptake system is responsible for the uptake of the drug.

Figure 3.38: The Arrhenius plots for the uptake of melarsen oxide by parent and resistant lines.




From the results obtained in Figure 3.36, the Arrhenius plots were derived as described under Materials and Methods (Section 2.25). The results presented are the logarithm of the rate of the reaction against the reciprocal of the absolute temperature. ●-● parent line; ■-■ resistant line.

Another observation from the temperature dependance experiment (Figure 3.37) is that the rate of transport of the drug in the parent line approached the rate of transport in the resistant line at low temperature. Therefore it is assumed that the total transport of the drug in the parent line is a complex system with a temperature and a non-temperature dependant component, or simple diffusion system.

In all cases the rate of transport were corrected for the extracellular drug component in the extracellular fluid (see Figure 3.10).

3.6.7 The thermodynamic characteristics of the transport systems in parent and resistant lines

To account for the effects of temperature on the rate of transport of the drug the phenomenon itself may be considered as a chemical reaction in which the active agent is taken from one side to the other side of a membrane (Stein, 1967). By using the Arrhenius equation it is possible to obtain some thermodynamic data inherent to the transport mechanism. Details of the calculation are presented in Materials and Methods. As suggested in the last section the rate of transport in the parent line approached the rate of transport in the resistant line at low temperature ($2^{\circ}C$) (see Figure 3.37). Therefore assuming that the diffusion component is also present in the parent line, it is possible to calculate the thermodynamic data inherent to the temperature dependant transport system by substracting the rate of transport found in the resistant line from the rate of transport in the parent line at the different temperatures investigated.

After correction for the diffusion component in the parent line and transformation of the data (rates of transfer in parent and resistant lines) using the Arrhenius plot, the results shown in Figure 3.38 were obtained. Calculation of the slopes of both straight lines allowed estimation of the energy of activation (Ea), enthalpy of activation ($\Delta H^{\ddagger}$), entropy of activation ($\Delta S^{\ddagger}$) and Gibbs free energy of activation ($\Delta G^{\ddagger}$) for the transition state (see Materials and Methods).

| | Parent | Resistant | Ratio |
|---|---|---|---|
| Ea (kJ $mol^{-1}$) | 59.71 | 19.33 | 3.09 |
| $\triangle H^{+}$ (kJ $mol^{-1}$) | 57.20 | 16.82 | 3.40 |
| $\triangle S^{+}$ (J $mol^{-1}$ $K^{-1}$) | 1164.66 | 515.76 | 2.26 |
| $\triangle G^{+}$ (kJ $mol^{-1}$) | -292.30 | -137.98 | 2.12 |

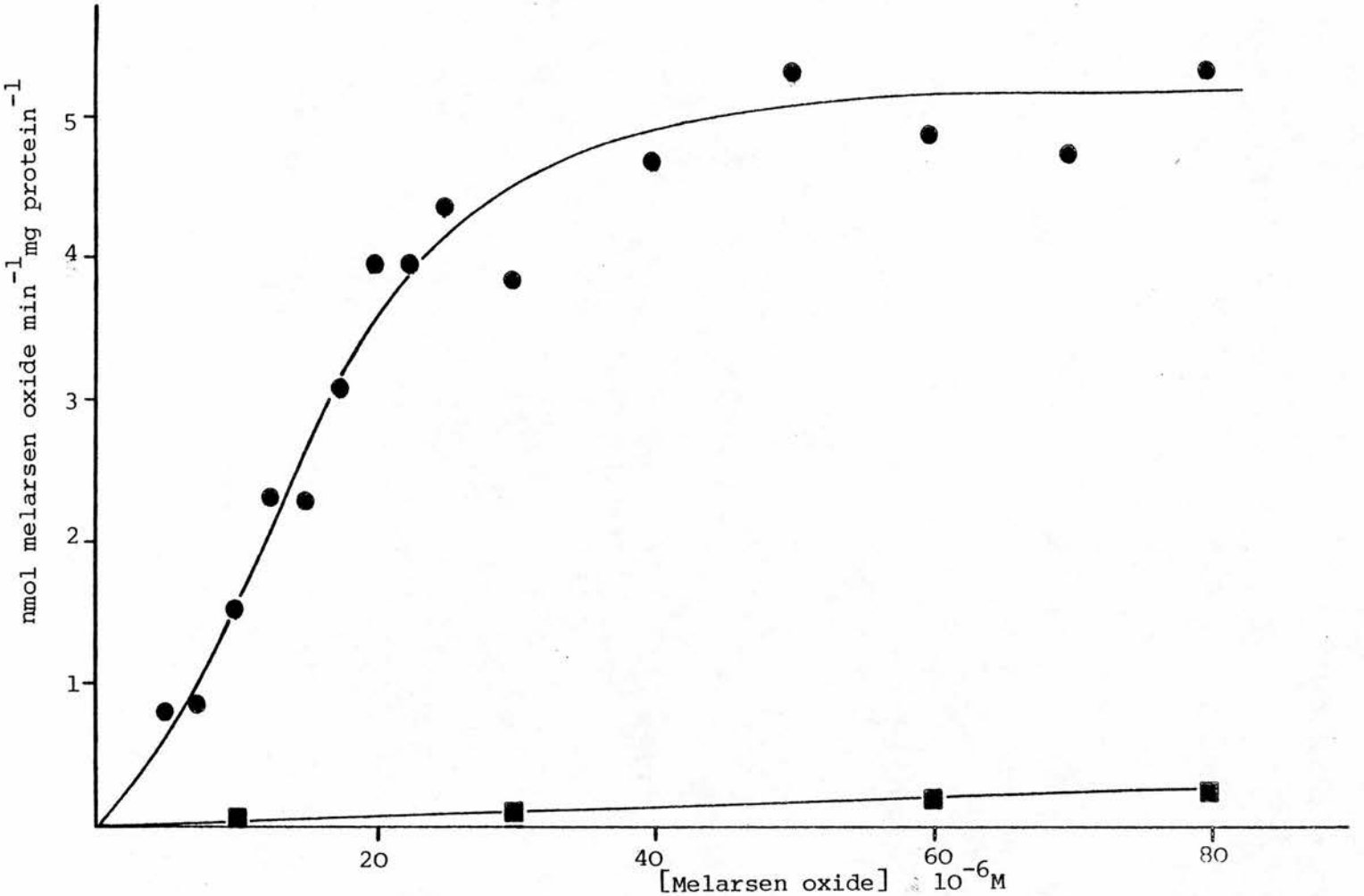
The results obtained indicate that changes have definitively occurred in the transport system of melarsen oxide as a result of the development of resistance.

Apparently two transport systems are involved, an energy dependant transport system in the parent line and a non-energy dependant diffusion mechanism, demonstrated to exist in the resistant line and assumed to be present in the parent line. It appears that the resistant line has apparently lost the energy dependant transport system or that it has been modified, and only a simple diffusion mechanism is operating for the transport of the drug as suggested by the low energy of activation obtained. With regard to the other thermodynamic parameters, the enthalpy, entropy and Gibbs free energy of activation, these are evaluated in more detail in the Discussion.

3.6.8 Concentration dependance of melarsen oxide uptake in parent and resistant lines

As mentioned in the Introduction most of the work done on the uptake of organic arsenicals has been about the relative drug uptakes of the different drugs tested, over long periods of time (Yorke et al., 1931; Hawking, 1937). Although this work has suggested the possibility of a reduced uptake in the resistant lines tested, the experiments design did not allow any definitive conclusion on the nature of the mechanism of transport. Hence no information exists on the nature of the change in the mechanism of transport during the development of resistance. Therefore, in order to understand the mechanism of transport in terms

Figure 3.39: The initial rate of uptake of melarsen oxide against the external drug concentration in parent and resistant lines.




All the experimental conditions were the same as in Figure 3.35, with the difference that the drug is estimated in the perchloric acid layer and the results are presented as a function of the extracellular concentration of melarsen oxide used in the experiments. ●-● parent line; ■-■ resistant line.

of the kinetics of the uptake of melarsen oxide a series of experiments were performed, as described below, on parent and resistant lines. Trypanosome suspensions were incubated in the oxygen electrode chamber at a temperature of 21°C. The highest concentration of drug used was $80 \times 10^{-6}$M melarsen oxide; this concentration was chosen from the motility experiment (Section 3.6.2) as at this concentration the trypanosomes remain motile after 60 seconds of incubation. The silicone layer filtration technique and the standard system for estimation of the drug were used as described in Materials and Methods.

The results of the influence of the concentration of melarsen oxide on the initial rates of uptake of the drug from three different experiments, for both parent and resistant lines are shown in Figure 3.39. In the first place a completely different pattern of drug uptake was found for parent and resistant lines. The possibility of a differential drug uptake as suggested in the metabolic section is corroborated here. It should be noticed that the short exposure (60 sec) of the trypanosomes to the drug ensures that the rates obtained are approaching the initial rates of uptake of melarsen oxide.

In the second place, the initial rates of uptake in the parent line as a function of the drug concentration approached a maximum value. Therefore a saturable carrier system is suggested for the transport of melarsen oxide in the parent line. This sustains the results of the last section as this pattern is typical of an energy dependant carrier mediated transport system.

Regarding the transport of the drug in the resistant line, low levels of drug uptake were associated with the trypanosomes after correction for the extracellular drug carried through. Although the concentration of the drugs are found at the low end of the calibration curve, an apparently linear rate of uptake was found. It is suggested that the

drug is probably taken by a phenomenon of simple diffusion, a suggestion substantiated by the low values of $Q_{10}$ and energy of activation obtained for the transport of the resistant line (see last section).

3.6.9 Kinetic parameters of the transport of melarsen oxide in parent and resistant lines

The information obtained from uptake experiments is often analysed in the same manner as the data from enzyme kinetics, the main objective being to show whether or not the data conform to the Michaelis-Menten type of kinetics (Neame and Richards, 1972). It was suggested by these authors that the graphical methods used to estimate the kinetic parameters of enzymatic reactions may be used to quantify the kinetic parameters of drug transport. However due to the scattering of the points, non-linear regression of the Michaelis-Menten function (Atkins and Gardner, 1977) and Hill equation (Atkins, 1973) were used to study the results obtained for the uptake of melarsen oxide in the parent line. It should be mentioned that these data were corrected by the diffusion component, supposed to be present in both lines of the parasite. Of the two computer programmes used the latter produced a better representation of the data obtained. The parameters estimated for the parent line were:

$K_t = 14.42 \times 10^{-6} M$

$V_{max} = 5.19$ nmoles $min^{-1}$ mg $protein^{-1}$

Hill coefficient = 2.27

($K_t$ is defined as the drug concentration needed to produce 50% of the maximal velocity for the transport of the drug.)

For the rate of uptake by the resistant line, a linear regression programme produced the following value:

$K_D = 3.1$ pmoles $min^{-1}$ mg $protein^{-1}$ /μM melarsen oxide

($K_D$ is defined as the diffusion constant for the transport of melarsen oxide in the resistant line.)

3.6.10 Concentration dependance of sodium melarsen uptake in parent and resistant lines

In general, pentavalent organic arsenicals are less toxic and less rapid in their action *in vivo* than the corresponding arsenoxides (Williamson, 1970). The possible lack of uptake of such pentavalent forms of organic arsenicals *in vitro* may be inferred from the work on cross resistance analysis of Williamson and Rollo (1959).

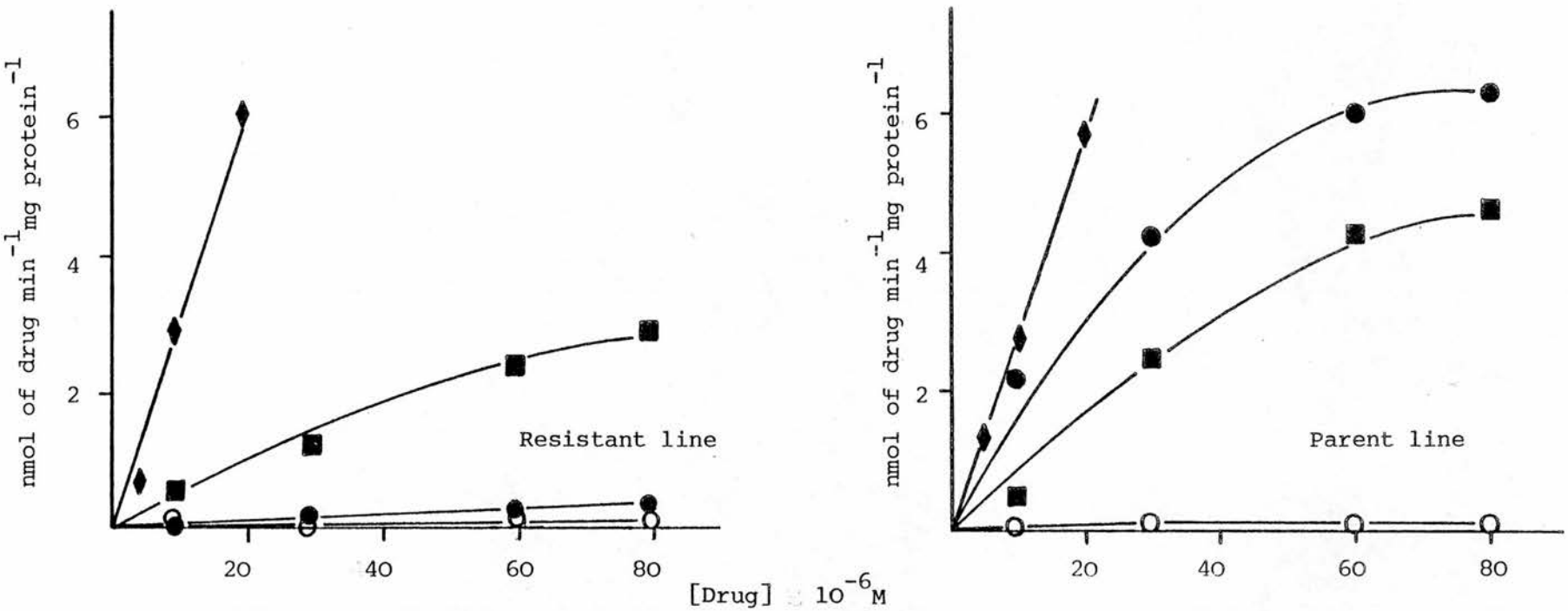
As for the trivalent arsenical drug little information exists on the mechanism of uptake of the pentavalent analogue. It is noteworthy that sodium melarsen is cross resistant *in vivo* with melarsen oxide (Williamson and Rollo, 1959). Therefore reduction before any biological effect is considered to be the first stage in the mechanism of action of pentavalent arsenicals (Lourie et al., 1935).

An experiment was designed to illustrate whether there was transport of the pentavalent drug in either line of trypanosomes. The results obtained for the uptake of sodium melarsen were different to those obtained with melarsen oxide, as it was observed that regardless of the concentration of sodium melarsen used there was no measurable uptake by either line of the parasites even after a period of incubation of 10 minutes. Hence, these results corroborate the need for extratrypanosomal reduction of the pentavalent arsenicals before they can be taken into the parasite.

3.6.11 The effect of different arsenicals on the motility of parent and resistant lines

Cross resistance studies have shown that trypanosomes made resistant to melaminyl arsenicals do not remain sensitive to neutral aromatic arsenicals (Williamson and Rollo, 1959). A study of the sensitivity of the newly developed resistant line to other arsenicals may help to understand the mechanism of development of resistance in terms of the structure of the drug.

Figure 3.40: The initial rate of uptake of different organic arsenicals against the external drug concentration in parent and resistant lines.




All the experimental conditions were the same as in Figure 3.35. The different organic arsenicals were estimated in the perchloric acid layer and presented as a function of the extracellular concentration of drug used. ●-● melarsen oxide; ■-■ para-aminophenyl arsenoxide; ♦-♦ phenyl arsenoxide; O-O inorganic arsenite.

Table 3.11: The effect of different arsenicals on motility in paren and resistant lines.

| | Time (minutes) | | | | | |
|---|---|---|---|---|---|---|
| Parent | 2 | 5 | 10 | 15 | 20 | 25 |
| control | +++ | +++ | +++ | +++ | +++ | +++ |
| melarsen oxide | ++ | ++ | + | + | +o | +o |
| reduced atoxyl | ++ | ++ | ++ | ++ | ++ | + |
| phenyl arsenoxide | ++ | + | +o | +o | +o | +o |
| sodium arsensite | +++ | +++ | +++ | +++ | +++ | +++ |
| Resistant | | | | | | |
| control | +++ | +++ | +++ | +++ | +++ | +++ |
| melarsen oxide | +++ | +++ | +++ | +++ | +++ | +++ |
| reduced atoxyl | +++ | +++ | +++ | ++ | ++ | + |
| phenyl arsenoxide | ++ | ++ | + | +o | +o | +o |
| sodium arsenite | +++ | +++ | +++ | +++ | +++ | +++ |

Trypanosomes from parent and resistant lines were isolated from blood elements as described in Sections 2.5, resuspended in PSGA buffer pH 8.0 (2.5 mg protein/ml approx.) and incubated with different organic arsenicals ($10 \times 10^{-6}$). The motility was evaluated as described under Materials and Methods (Section 2.7).

The arsenicals used were: arsenite, phenylarsenoxide, para-amino phenyl arsenoxide and the already tested melarsen oxide; the organic arsenicals used may be considered to be structural analogues of the melaminyl drug (see Table 1.2).

A preliminary motility experiment was carried out, the trypanosomes being incubated under the standard conditions (see Materials and Methods) with $1 \times 10^{-5}$ M of the various drugs. The motility was monitored under phase contrast microscopy at different times after the addition of the drugs, the results are shown in Table 3.11. It is inferred from these results that the melarsen oxide resistant line of T. brucei is still as sensitive to para-aminophenyl arsenoxide and phenyl arsenoxide as the parent line. Inorganic arsenite did not show any effect on either line under the conditions used. Therefore it is suggested that the resistant character is apparently developed against the melaminyl ring of the drug.

3.6.12 The uptake of different arsenicals by parent and resistant lines

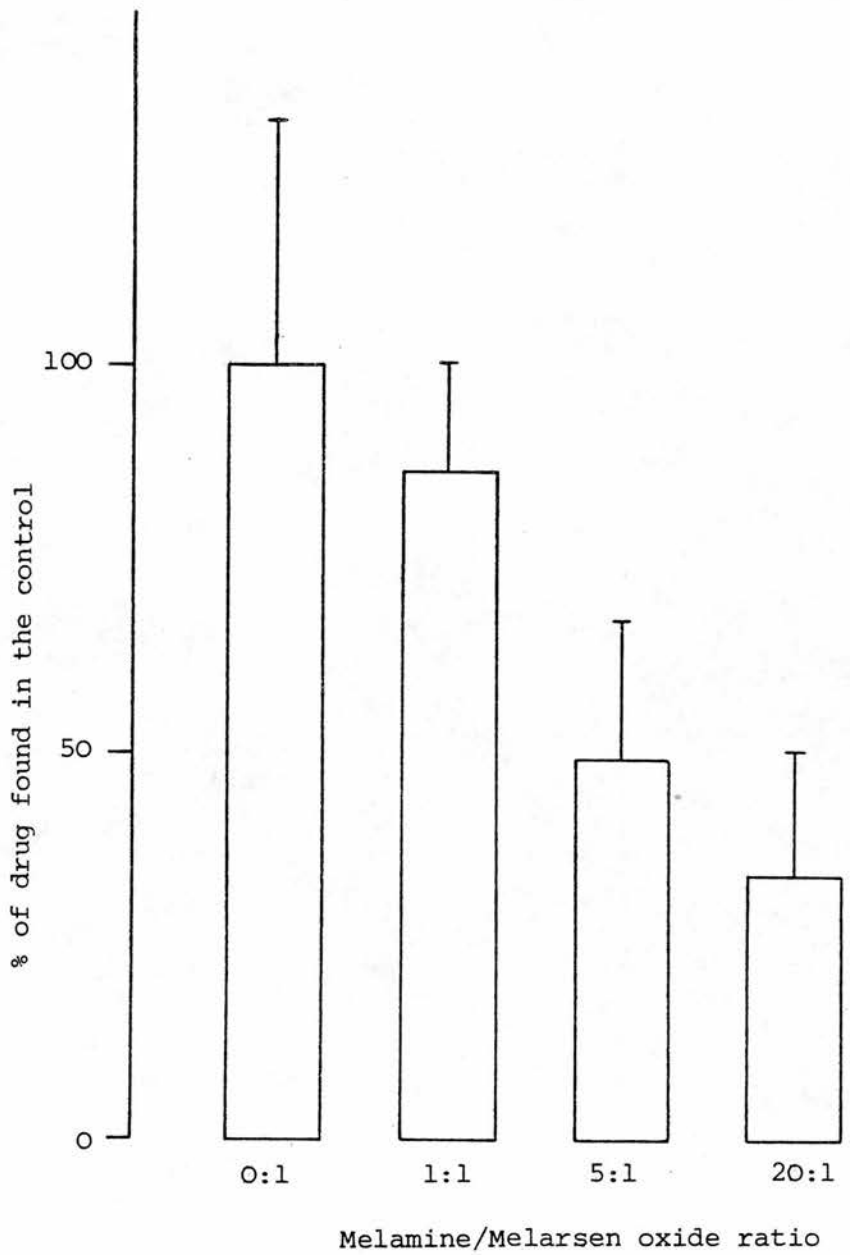
In order to corroborate the suggestion made of the possible development of resistance towards the melamine residue in the molecule of melarsen oxide, an uptake experiment was performed using the different arsenicals mentioned in the previous section. All the standard conditions for incubation and exposure of the trypanosomes, and estimation of arsenicals were used (as reported in Materials and Methods).

The results are shown in Figure 3.40. It was found that both lines take up phenylarsenoxide very actively. Reduced atoxyl penetrates the resistant cells less readily than the parent cells.

Regarding the kinetic parameters for the transport of para-amino-phenyl arsenoxide and phenyl arsenoxide in parent and resistant lines, the following data were obtained after linear transformation (s/v vs S) of the initial velocities as a function of the substrate concentration:

Figure 3.41: The effect of melamine on the transport of melarsen oxi

in the parent line.




Trypanosomes from parent line were prepared and resuspended in buffe
as in Figure 3.85. The cells were incubated with a fixed concentrati
of melarsen oxide ($80.10^{-6}$ M) and different concentrations of melamine
for 60 seconds. Aliquots (0.5 ml) were centrifuged using the silicon
layer filtration technique and the drug estimated in the perchloric a
layer. Details of the methods are described in Materials and Methods
and in the text. The results are presented as percentages of the
uptake in the absence of melamine (0:1).

| | Parent | | Resistant | |
|---|---|---|---|---|
| | $K_t$ | $V_{max}$ | $K_t$ | $V_{max}$ |
| - Para-aminophenyl arsenoxide | 81.05 | 9.5 | 182.6 | 9.1 |
| - Phenyl arsenoxide | Not calculated as the uptake was apparently linear in the $V_o$ vs S plot. However the rate of transport are apparently the same in parent and resistant lines. | | | |

($K_t$ = μM; $V_{max}$ = nmoles $min^{-1}$ mg $protein^{-1}$).

3.6.13 The effect of melamine on the transport of melarsen oxide

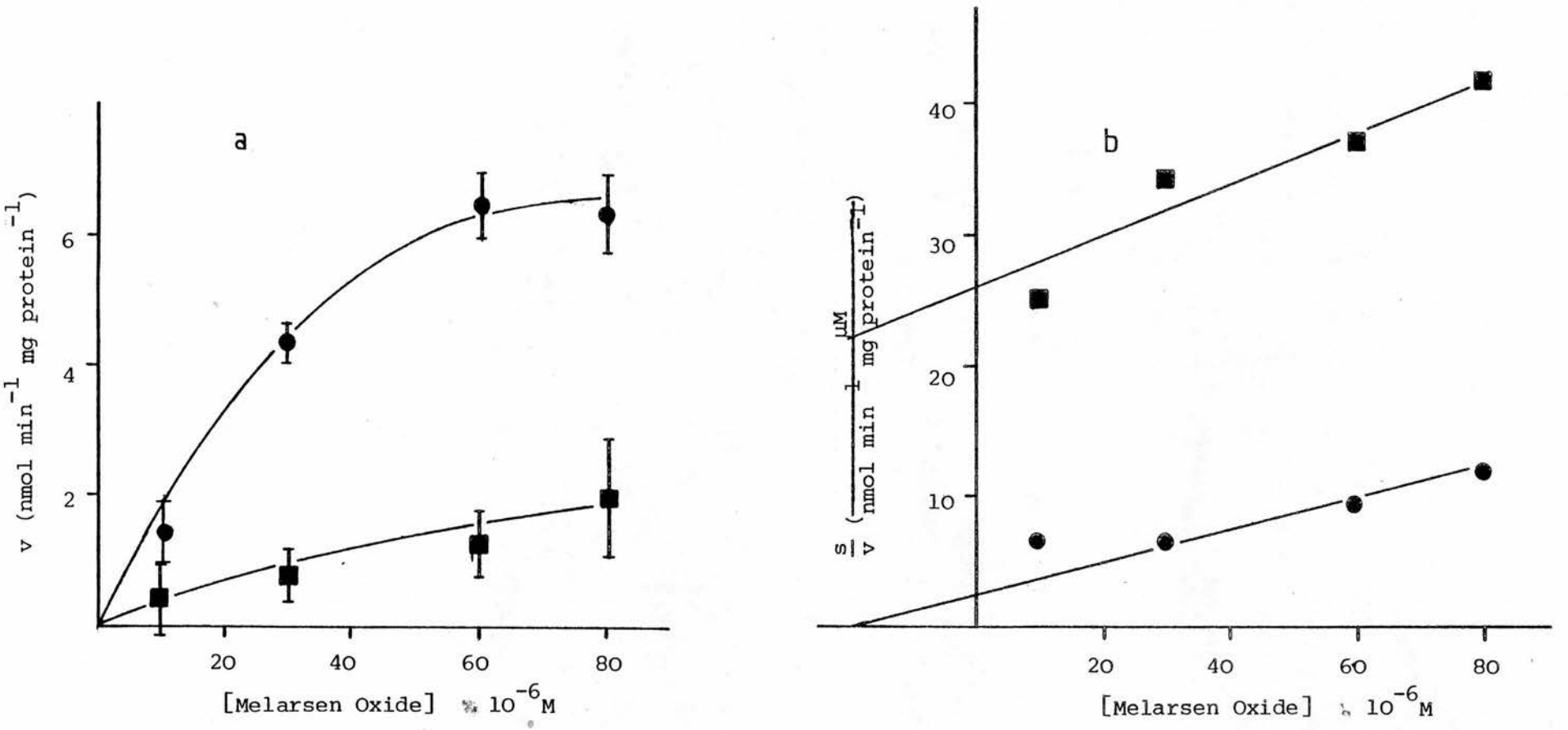
From the studies on the selective reversal of the action of melaminyl arsenicals by melamine *in vivo* and *in vitro*, it has been inferred that melamine interferes with the initial binding of the drug to *T. rhodesiense* (Williamson, 1959a). However, little is known on the mechanism by which the structure inhibits the action of the melaminyl drugs. Furthermore any inhibition produced by melamine can be used to illustrate the specificity of the transport mechanism of the melaminyl drugs as the triazine ring is a structural analogue of the active agents.

Some experiments were designed to investigate this point. The standard conditions of incubation and exposure of the parasites using the silicone layer filtration technique were used. Trypanosomes from the parent line were incubated with different concentrations of melamine (from a freshly prepared solution in warm buffer) for 30 seconds prior to the addition of melarsen oxide to a final concentration of $80.10^{-6}$M. Samples were taken in triplicate and analysed for arsenic; the results are shown in Figure 3.41. It was found that the inhibition of drug uptake increased with the concentration of melamine.

3.6.14 The influence of melamine on the uptake of melarsen oxide in parent line

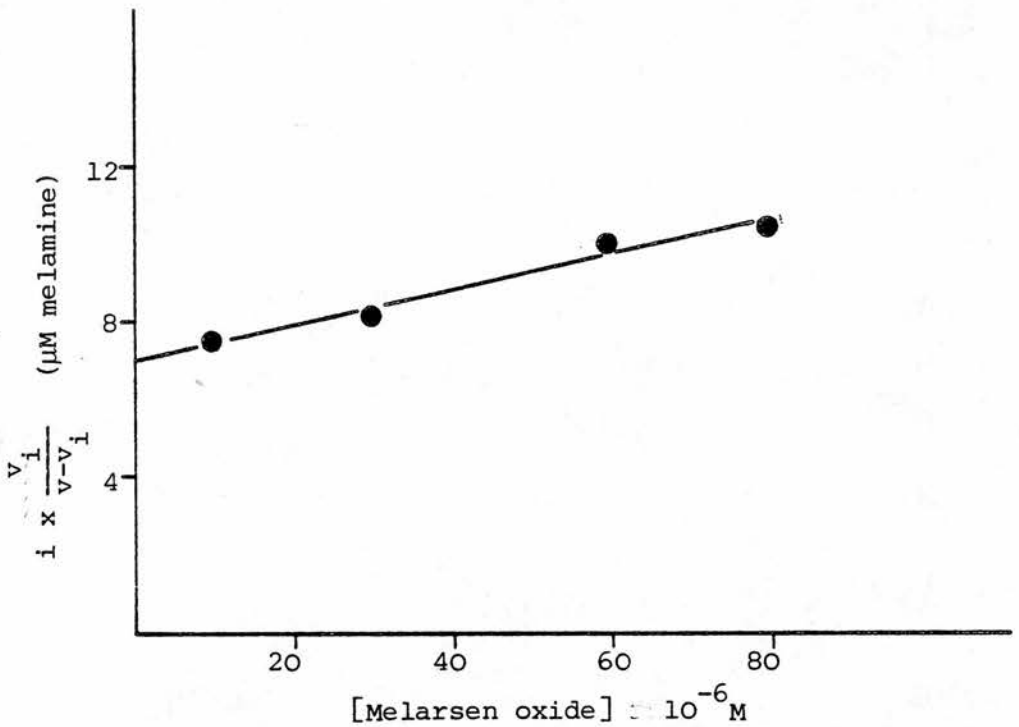
It was of interest to investigate the type of mechanism involved in the inhibition of melarsen oxide uptake. The design of the next experiment

Figure 3.42: The effect of melamine on the initial rates of transfer of melarsen oxide in the parent line.




Trypanosomes isolated from blood elements as described in Section 2.5 were resuspended immediately before incubation in PSGA buffer pH 8.0 with and without melamine (30 x $10^{-6}$M). After incubation for 60 sec. at 21$^{o}$C of both suspensions of trypanosomes with different concentrations of melarsen oxide, aliquots (0.5 ml) were centrifuged using the silicon layer filtration technique and the drug was estimated in the perchloric acid layer. The results are presented as the initial rate of transport as a function of melarsen oxide

Figure 3.43: The estimation of the inhibitory constant ($K_i$) for transport of melarsen oxide with melamine as the inhib




The results shown in Figure 3.42 were analysed using the Hunter and Downs method and the values for the fractional velocities obtained, in the presence of inhibitor (melamine 30 x $10^{-6}$M), are presented as a function of the melarsen oxide concentration (see text for deta

is similar to the one used to study the concentration dependance of the uptake of the drug in Section 3.6.8, the only difference being that melamine is present in the buffer to a final concentration of $30.10^{-6}$M in one set of experiments.

The results of the influence of inhibitor on the uptake of melarsen oxide are shown in Figure 3.42a. The data is presented as the initial rate of uptake with and without melamine as a function of melarsen oxide concentration. To illustrate the type of inhibition produced, a linear transformation of the data (s/v vs S) is used; the results are shown in Figure 3.42b. The pattern found is consistant with the suggestion of competitive inhibition. Perfect parallel lines are not found, hence a small mixed inhibition component is present affecting the $V_{max}$ of the transport system when melamine is present.

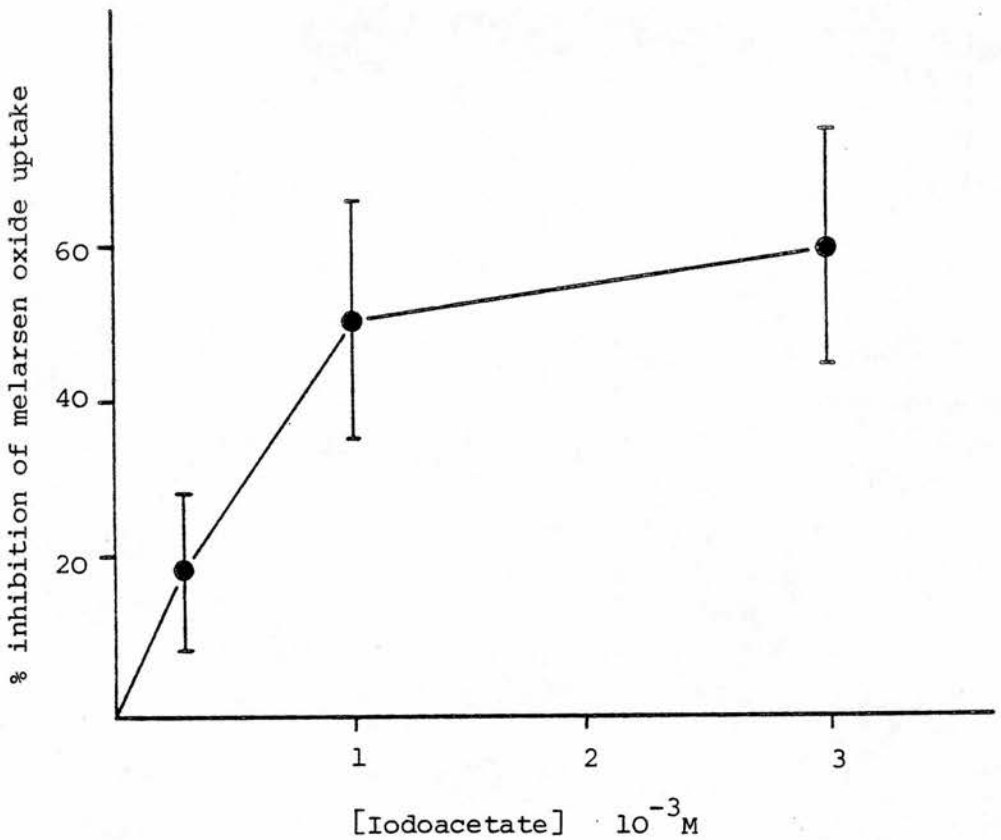
It may therefore be suggested that the transport mechanism for melarsen oxide is specific for the melaminyl part of the drug. This data on the effect of melamine on the uptake of melarsen oxide can also be used to estimate the $K_i$ value (inhibitory constant) of the former for the drug. The method of Hunter and Downs (1945) is used for this purpose (see Materials and Methods).

Figure 3.43 shows the results obtained when the fractional velocity in the presence of the inhibitor is plotted as a function of the drug concentration. The straight line obtained intersects the y axis at the $K_i$ value, found to be $7.0 \times 10^{-6}$M melamine under the conditions used.

3.6.15 The effect of iodoacetate on the uptake of melarsen oxide by parent line

There are some difficulties in demonstrating the energy dependance of an active or uphill transport mechanism, as the coupling to the energy yielding process is not always direct. Furthermore the

Figure 3.44: The effect of iodoacetate on the uptake of melarsen ox
in parent line.




Trypanosomes were isolated from blood elements using the standard tec
(Section 2.5). The cells were resuspended in PSGA buffer pH 8.0 and
incubated at 21°C with different concentrations of iodoacetate, prior
addition of melarsen oxide (30 x $10^{-6}$M). Aliquots (0.5 ml) were cent
and the drug estimated as described under Materials and Methods
(Sections 2.15, 2.16 and 2.19). Cells were checked for viability at
end of the experiment. The results are presented as percentage of
inhibition compared to untreated trypanosomes as a function of iodoac
concentration.

metabolic poisons used for this purpose may produce non-specific changes in the membrane that could generate artifacts in the flux of the substrate being studied (Goldman, 1973).

In spite of these difficulties an experiment was performed to show the possible effect of iodoacetic acid, a classical metabolic inhibitor (Lehninger, 1975) which has been reported as being toxic to the metabolism of trypanosomes (Williamson, 1959b).

Trypanosomes were incubated under the standard conditions (see Materials and Methods) at a temperature of 21°C with different concentrations of iodoacetate (0.3; 1.0 and 3.0 x $10^{-3}$M) for 30 seconds. After this period melarsen oxide was added to a final concentration of 30 x $10^{-6}$M and incubated for a further 60 seconds. The centrifugation and estimation of the drug was carried out as described in Materials and Methods.

The results of the residual uptake of melarsen oxide when compared with the uptake in iodoacetate untreated trypanosomes are in Figure 3.44. It is suggested that the mechanism of transport of melarsen oxide in the parent line is energy dependant under the conditions described as the trypanosomes were 100% motile at the end of the incubation with IAA and melarsen oxide.

3.6.16 Summary

It was suggested from the metabolic experiments that the development of resistance in the new line of T. brucei was probably associated with a permeability barrier for the drug. However the suggestion at that stage did not discard the possibility of modified target enzymes. As a result of this, PK a focal point of arsenical action and PGK a similar enzyme, as function is concerned, were examined and apparent differences were not found. Therefore the permeability properties of melarsen oxide were investigated in parent and resistant line organisms.

The conditions were set up to ensure a short exposure of the trypanosomes to the drug, a necessary requirement to have good estimates of the initial rates of uptake of the drug in both lines. The silicone layer filtration technique was used for this purpose, a method which has already been used for studies of drug uptake by trypanosomes, as mentioned at the beginning of this section. According to the concentration of trypanosomes used for incubation (c.a. 5.0 mg protein/ml) the conditions were: 60 seconds incubation at a maximum of 80 x $10^{-6}$M melarsen oxide in the exposing solution, and 60 seconds centrifugation at approximately 10,000 g for complete separation of the parasites from the exposing solution. The extracellular fluid value of 12.56 $\pm$ 0.72 µl per 2.5 mg of trypanosomal protein was calculated using $^{14}$C inulin as an extracellular marker. This value was used to correct for the extracellular drug associated with the uptake experiments of this section.

A chemical method for the estimation of arsenic in the drug was used in these studies as an assay system for total drug.

The first evidence of a different mode of uptake of melarsen oxide by parent and resistant line organisms was obtained from the time course experiments at different temperatures. It was found that the parent line has a large $Q_{10}$ value (2.0), i.e. a temperature dependant transport system for melarsen oxide. However the resistant line has a low $Q_{10}$ value (1.2), i.e. a non-temperature dependant transport system for the drug; this low $Q_{10}$ transport mechanism seems to be present as well in the parent line as its rate of transport approached the rate of the resistant line at low temperature. The thermodynamic data calculated from these experiments using the Arrhenius equation showed an $Ea^{\ddagger}$ value of 59.71 and 19.33 kJ $mol^{-1}$ for parent and resistant lines respectively. These data corroborate that the high rate of transport may be due to a carrier mediated system in the parent line, and a simple diffusion alone

for the drug transport in the resistant line. Further evidence in this respect was obtained from the concentration dependance experiments, where a carrier mediated system is suggested for the parent line with a $K_t$ value of 14.42 x $10^{-6}$M melarsen oxide and a $V_{max}$ value of 5.19 nmoles $min^{-1}$.mg $protein^{-1}$. The transport system appeared to be complicated as the Hill equation was the mathematical model able to explain the data obtained.

With regard to the resistant line a low rate of diffusion was obtained with a $K_D$ value of 3.1 pmoles $min^{-1}$.mg $protein^{-1}/10^{-6}$M melarsen oxide.

From the uptake studies of other related arsenicals it was found that the resistance is mainly developed against the melaminyl residue of melarsen oxide. Further experiments on the mechanism of inhibition of the transport of melarsen oxide by free melamine suggested that the latter inhibits by competing with the drug with a $K_i$ value of $7.0.10^{-6}$M melamine. This in turn suggests that the melaminyl residue is vital for the uptake or in other words that the mechanism of transport shows some specificity for this part of the drug. Iodoacetate inhibition of the uptake of melarsen oxide suggests the possibility of energy dependance in the transport of the drug in the parent line.

# 4. DISCUSSION

## 4.1 Discussion of the studies on phosphoglycerate kinase

As mentioned in the Introduction, the kinases are considered to be the main targets of organoarsenical drugs in trypanosomes. Some of the enzymes in this group have been studied in certain detail with respect to their sensitivity to these drugs (Table 1.3). PGK was the least studied enzyme of the group. Evidence has been presented in this thesis that the enzyme is in fact inhibited by different arsenicals, including a member of the melaminyl series. Furthermore, the similar function of this enzyme to PK in substrate level phosphorylation and the known nature of the latter as a target for organoarsenical drugs (Flynn and Bowman, 1974), suggested a potential participation of PK or PGK in the development of resistance. However, it was found that this was not the case for either enzyme.

In spite of the lack of participation in the development of resistance, the fact that the enzyme was inhibited by arsenicals was considered of sufficient importance to study the enzyme in more detail, as basic knowledge on the general mechanism of action of organoarsenicals can be used in a rational approach to the development of new trypanocides.

### 4.1.1 The general properties of phosphoglycerate kinase from T. brucei

The pH profile observed for the trypanosome enzyme (Figure 3.18), showed maximal activity between 6.5-8.2. This range is not as great as the pH range of PGK from other sources. It was found, for example, that the range of the rabbit muscle and yeast enzymes was approximately between pH 6.0-9.2 (Kritsch and Bücher, 1970) and for the pea seed enzyme, pH 6.7-9.2 (Axelrod and Bandurski, 1953). The biological interpretation of pH profiles is complex, as assumptions have to made with regard to the physicochemical characteristics of the enzyme that are not necessarily valid (Cornish-Bowden, 1979). However, the localisation of the trypanosome enzyme in the glycosome (Opperdoes and Borst, 1977; Oduro, 1977)

may suggest that PGK is in a rather specialised environment where changes in pH could play an important role in the control of enzyme activity.

It has been reported that PGK is activated by $Mg^{2+}$ and $Mn^{2+}$ ions (Larsson-Raznikiewicz, 1964, 1967; Rao and Osper, 1961). The possible function of the metal ion has been studied and it is likely that the divalent cation complexes of ATP and ADP are the true substrates of the reaction (Larsson-Raznikiewicz, 1964, 1967).

It was found in this work that $Mg^{2+}$ and $Mn^{2+}$ are also activators for the trypanosome enzyme (Figure 3.19). In the case of $Mn^{2+}$, the ion had an inhibitory effect at concentrations above $5 \times 10^{-3}$M. A similar inhibitory effect has been observed in studies of the metal ion specificity of yeast PGK (Larsson-Raznikiewicz, 1970a). This author found that at low concentration, this ion is a competitive inhibition with respect to $MnATP^{2-}$. However as the concentration of the free cation increases the inhibitory effect becomes uncompetitive. It was also found by the same author that the uncompetitive inhibition is caused by complexing of $Mn^{2+}$ to the cosubstrate G3P the complex being suggested as the true inhibitor. Therefore it is concluded that the inhibition effect observed for $Mn^{2+}$ on trypanosomal PGK may be produced by a similar mechanism.

With regard to the nucleotide specificity of PGK it has been found that yeast and muscle PGK are not specific as the phosphate donor for the back reaction. Hence ATP, ITP, and GTP support similar levels of activity (Krietsch and Bucher, 1970). However this concept is not accepted by all authors as it has been reported that rabbit muscle PGK is absolutely specific for ATP, ADP, G3P and 1.3-diphosphoglycerate (Rao and Osper, 1961). The results obtained on the nucleotide specificity of the trypanosome enzyme showed it to be rather specific for ATP with only residual activity with CTP and UTP. Negligible activity

was found with GTP as the cofactor (Table 3.6). The biological significance of this finding is difficult to appraise. However this enzyme is situated inside the glycosome which is self-sufficient in keeping the ATP/ADP balance. It may be inferred that the specialisation of the particle may have favoured the development of a variant PGK which is more specific for its phosphate donor.

4.1.2 The kinetic properties of phosphoglycerate kinase from T. brucei

The kinetic properties of PGK from different sources have been investigated recently, using the back reaction of the enzyme, coupled to the oxidation of NADH by glyceraldehyde phosphate dehydrogenase (Scopes, 1973). When the interaction of ATP and G3P was similarly studied with the trypanosome enzyme, it was found that when varying the concentration of either substrate and keeping the cosubstrate concentration constant apparent hyperbolic responses were obtained for the activity of the enzyme as a function of the variable substrate concentration (Figures 3.21 and 3.23). After transforming the data using the equation proposed by Hanes (1932) biphasic linear responses were found for ATP as the variable substrate and a single linear response for G3P (Figures 3.22 and 3.24). Similar observations have been reported on the binding of the nucleotide to PGK from different sources (Yoshida and Watanabe, 1972; Schierbeck and Larsson-Raznikiewicz, 1979; Scopes, 1978a,b). It has been suggested that the enzyme does not follow the typical Michaelis-Menten pattern and that two binding sites for ATP exist on the enzyme, with different kinetic constants. Regarding the Michaelis-Menten constants; the two $K_m$ values reported for ATP in trypanosome PGK (Table 3.8) are similar to those suggested for human erythrocyte PGK (Yoshida and Watanabe, 1972). The $K_m$ value for G3P however was higher than the respective $K_m$ value for the enzyme described by the latter authors.

On the function of the two binding sites, it was proposed that the low affinity binding site acts as a regulatory site (Larsson-Raznikiewicz and Schierbeck, 1977) and that the other binding site acts as the only catalytic site of the enzyme (Schierbeck and Larsson-Raznikiewicz, 1979).

In view of the evidence gathered on PGK from other sources and because of the similarities to the binding characteristics of the nucleotide in the trypanosome enzyme it is suggested that the trypanosome enzyme is similar to PGK from other sources with respect to the number of nucleotide binding sites and their functions.

Regarding the binding of G3P at a constant concentration of the nucleotide a hyperbolic function was obtained and after transformation of the data, a simple linear response was observed (Figure 3.24). One binding site is therefore suggested for this substrate in the trypanosome enzyme. In this respect the parasite PGK is similar to human red cell PGK as it has been reported that this enzyme has only one site for G3P (Yoshida and Watanabe, 1972).

When the data obtained from the kinetic studies were transformed using the Hill equation (Hill, 1910) (Figure 3.25) it was noticed that the fractional velocity as a function of log$_{10}$ ATP concentration at constant concentration of G3P was biphasic, with a Hill coefficient (n values) of less than one at low concentrations of ATP and an n value of about one at higher substrate concentrations. This phenomenon can be interpreted in terms of negative cooperativity at low substrate concentrations with subsequent activation by higher substrate concentrations. The results obtained with respect to G3P using the Hill equation did not suggest any cooperative effects. Similar conclusions to those presented here can be drawn from experiments performed with yeast PGK (Schierbeck and Larsson-Raznikiewicz, 1979). Substrate activation has also been

found in erythrocyte PGK when studying the binding of substrates in the forward reaction (Ali and Brownstone, 1976).

The biological significance of the change in the n value described for trypanosomal PGK is difficult to appraise, and more data is needed to clarify the situation, preferably, with a purer preparation of the enzyme. However, the bilobular model suggested for horse muscle PGK from data obtained using X-ray crystallography (Blake et al., 1972; Banks et al., 1979) may be used to visualise the situation. These studies showed that two domains are found in the monomeric enzyme. Therefore the possibility, that binding of the nucleotide to one domain may alter the catalytic properties of the other domain, is apparently feasible.

4.1.3 The mechanism of action of phosphoglycerate kinase from T. brucei

The mechanism of action of PGK has been studied on the yeast and human erythrocyte enzymes (Larsson-Raznikiewicz and Ardvison, 1971; Lee and O"Sullivan, 1975). These authors suggest that the enzyme has a sequential mechanism from kinetics studies in the absence of the products. These studies, however, did not discriminate between an obligatory order of addition and release of reactants, and combination in random order. Product and substrate analogue inhibition experiments carried out by the same authors finally defined the system as a rapid equilibrium random-type mechanism. These results are corroborated by recent experiments of Schierbeck and Larsson-Raznikiewicz (1979).

When an attempt was made to illustrate the mechanism of action of trypanosomal PGK using the back reaction of the enzyme, a very complex picture was obtained because of the biphasic linear nature of the primary plots. In the first primary plot used (1/v vs 1/s) (Figure 3.26b) to show the binding of ATP at different concentrations of G3P, it was observed that the binding of ATP was apparently independant of the concentration of the cosubstrate as the lines obtained converged at the

same values of low and high $K_m$ values. However when using a second primary plot (s/v vs S) (Figure 3.26c) a small competitive component was observed with respect to the low affinity binding site of the enzyme. One possible explanation for these deviations is the presence of free $Mg^{2+}$ ions in the assay system which might act as a competitive inhibitor with respect to $ATPMg^-$ (Larsson-Ranikiewicz, 1964).

With regard to the effect of the concentration of ATP on the binding of G3P, it was observed that there is, an apparent effect on the $K_m$ for G3P (see primary plots (Figure 3.27)). It is therefore suggested that ATP may produce a change in conformation in the enzyme to allow for the different affinities found for G3P.

In general the mechanism of trypanosomal PGK was found to be complex. The possibility of a sequential mechanism, where the binding of substrates occurs prior to the release of the products may be allowed. However no definitive conclusion can be reached as it was found that free divalent cations ($Mg^{2+}$) have a possible inhibitory effect as reported above. Furthermore the binding of G3P at different concentrations of ATP produced anomalous results.

4.1.4 The effect of melarsen oxide on phosphoglycerate kinase from T. brucei

As reported in the Introduction, arsenical drugs are general inhibitors of different enzymatic reactions, the main common factor being that these drugs are regarded as thiol inhibitors. Although the reaction of the metal moiety accounts for the lethal action, the organic residue explains the structural specificity of the drug for the parasite (Williamson, 1970).

It can be seen from Table 1.3 that the kinases are the most sensitive group of enzymes in trypanosomes, due apparently to their thiol character. With respect to the focal point of action of arsenicals in

the parasites, different alternatives have been prepared. It was originally suggested that the action of these drugs was due to the specific action on hexokinase (Chen, 1948; Marshall, 1948). The evidence was not however accepted by other authors (Cantrell, 1953). The situation was finally clarified by Flynn and Bowman (1974), who suggested pyruvate kinase as the primary focal point of action in the parasite.

Although evidence has been presented in this work that PGK is also sensitive to inhibition by arsenicals (Figure 3.32), the metabolic evidence (Section 3.3) indicates that PK is still a major focal point of action in the resistant line of T. brucei.

When the effect of melarsen oxide on PGK was studied, it was found that the inhibition produced by the drug was directly related to the binding of the different substrates. Variation of ATP concentration with and without the drug showed a typical non-competitive inhibition with a considerable reduction in the $V_{max}$ value. The $K_m$ values are apparently independant of the presence of the drug (Figures 3.29). It must be pointed out that the primary plot showed a typical linear biphasic response in the presence of the drug. It is concluded that melarsen oxide acts in a similar way at both binding sites producing the non-competitive inhibition mentioned above. When the effect of the drug was studied on the binding of G3P (Figure 3.30) a different pattern of inhibition emerged, producing an increase in the apparent $K_m$ value for G3P. It appears, therefore, that melarsen oxide is competing for the G3P binding site.

With respect to the general mechanism of action of melaminyl arsenicals, a great similarity is found when comparing the patterns of inhibition of PGK and PK (Flynn, 1971). It appears that melarsen oxide acts by a general non-competitive mechanism regarding the nucleotide and by a competitive effect with the cosubstrate. This

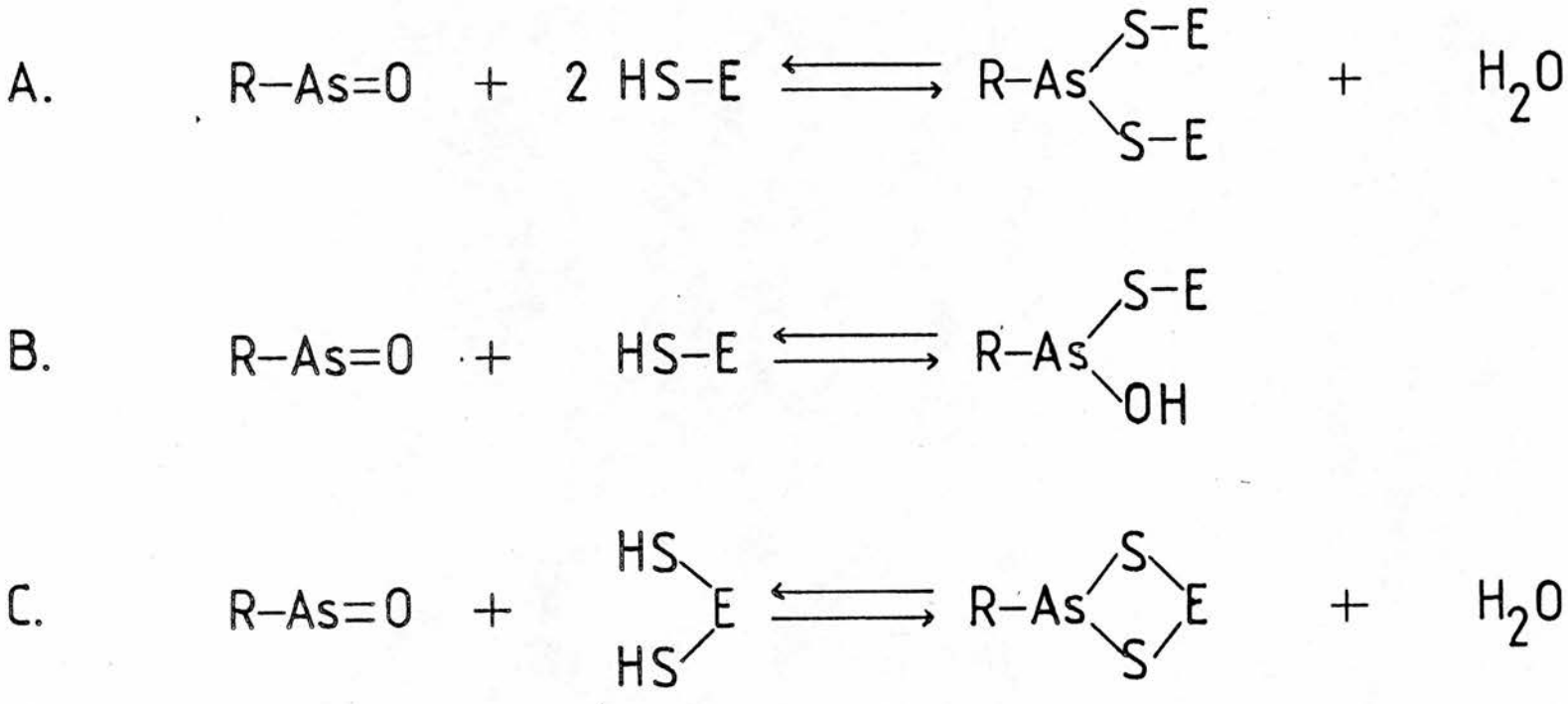
similarity is probably widespread as it has been found that most of the kinases studied by X-ray crystallography have active sites with a similar spatial structure (Anderson et al., 1979).

Reversible inhibition in general is characterised by an equilibrium between enzyme and inhibitor. The equilibrium constant ($K_i$) defines the effectiveness of the inhibitor (Dixon and Webb, 1964). These authors proposed that the reversible character also applies to inhibitors which do not dissociate spontaneously but which can be removed by a suitable chemical reaction. The nature of the thioarsenite interaction is not definitively settled but it has been suggested that the relatively stable interaction between trivalent arsenicals and -SH groups is readily reversed (Webb, 1966). $K_i$ values reported for other enzymes which are inhibited competitively with respect to their substrate tend to have a wide range of variation; liver aldehyde oxidase is inhibited by arsenite with a $K_i$ value of $3 \times 10^{-3}$M indicating a rather low affinity of the enzyme for the inhibitor (Palmer, 1962), when compared with the same enzyme in rabbit liver which is inhibited competitively but with a $K_i$ value of $0.0063 \times 10^{-3}$M (Rajagopalan and Handler, 1964).

The $K_i$ values for melarsen oxide were calculated on the assumption that the interaction of the drug and the enzyme is reversible under the condition described above; the values were calculated with the method of Dixon (Figure 3.31). The low $K_i$ values found in particular with respect to G3P imply that melarsen oxide appears as a good inhibitor of the trypanosomal enzyme.

Trypanosome PGK was not only sensitive to melarsen oxide but also to other organic arsenicals. The efficiency of each of the arsenicals tested is described in terms of the amount of drug required to produce 50% inhibition ($I_{50}$ values). It was found (Figure 3.32) that the order of efficiency was melarsen oxide>phenyl arsenoxide>para-aminophenyl arsenoxide.

Figure 4.1: The various possible modes of interaction between monosubstituted arsenicals and thiol groups on enzymes.




E = Enzyme

R = Residue

The pentavalent sodium melarsen does not produce inhibition under the conditions used and neither arsenite nor melamine are inhibitors of the enzyme.

In general the results obtained for the inhibition of PGK were similar to those presented for the inhibition of PK (Flynn, 1971) which was not inhibited by inorganic arsenite either but very effectively by monosubstituted arsenicals similar to those used in this thesis.

At this stage it is interesting to consider the nature of the interaction between monosubstituted arsenoxides and -SH groups in enzymes as it will help to rationalise the results obtained. Of the alternatives proposed (Figure 3.45) reaction A appears to be unlikely because of the steric and electrostatic impediment of bringing two enzyme molecules together by the arsenic moiety of the drug. The other two reactions B and C can be considered as viable alternatives to explain the interaction with monosubstituted arsenicals (Webb, 1966). Reaction B can occur but if it does, it may not produce a very efficient binding in terms of the rather more stable cyclic thioarsenite formed in reaction C.

Although no evidence is available about the number of -SH groups at the active site of PGK some suggestions are made according to the results presented by other authors. By analogy to the similar inhibition found with respect to PK (Flynn, 1971) there are some main observations that can help to determine the interaction of PGK with organic arsenicals: 1) the competitive nature of the inhibition with respect to G3P produced by melarsen oxide, 2) the lack of inhibition by inorganic arsenite and 3) the lack of inhibition by either melamine or pentavalent sodium melarsen which suggests the involvement of -SH groups for the inhibition of trypanosome PGK.

Alternative C (in Figure 4.1) suggests that the cyclic thioarsenite formed in this reaction may be stable enough to resist any competition

by G3P, hence a primary stage of interaction with the enzyme must take into account the possibility of a reversible interaction with PGK to allow for the competition effect with G3P. Therefore alternative B, forming a less stable, readily hydrolisable compound may be inferred to explain the results obtained.

As observed in this thesis (Figure 3.35) the interaction of melarsen oxide with trypanosome protein is relatively stable as most of the drug was found bound to the denatured protein even under strong acid conditions; thus the possibility of a more stable interaction may be formed after the initial stage (B) mentioned above. This suggestion implies the possibility of time dependant changes in the inhibition produced by the drug. This specific area was not investigated in this work however results presented by Peters and Sanadi (1961) showed the xanthine oxidase is protected against arsenite by the substrate but that the final inhibition is non-competitive with respect to xanthine. A similar possibility was also proposed to explain the final inhibition of trypanosome PK (Flynn, 1971).

The results presented by other authors with respect to the differential inhibitory power of arsenite and monosubstituted organic arsenicals observed in other enzymes may help to settle the final mechanisms of interaction of the drug with -SH groups in enzymes. Succinate oxidase was inhibited differentially by inorganic arsenite and monosubstituted organic arsenicals (Barron and Singer, 1945; Peter et al., 1946). At the same time it is considered that inorganic arsenite has a greater affinity for dithiol groupings as the drug readily reacts with dimercaprol (2,3-dimercaptopropanol) but not with the monothiol glutathione (Aldridge and Gemer, 1955), furthermore no mercapeptide formation was observed even at 100 x $10^{-3}$ M arsenite (Drummond and Stern, 1960). Strong inhibition by inorganic arsenite as the one

reported for pyruvate oxidase from pigeon brain (Peter et al., 1946) suggests the possibility of a vicinal dithiol in the enzyme. Neither trypanosome PGK as observed in this work nor PK (Flynn, 1971) were inhibited by inorganic arsenite therefore by comparing these observations it is possible to suggest that the initial interaction with the trypanosome enzyme occurs via a monothiol and upon binding with the drug a change in conformation of the enzyme may allow the formation of a more stable thioarsenite.

Finally the inhibition of trypanosome PGK with monosubstituted arsenicals confirms the thiol character of the enzyme when compared with rabbit muscle PGK which is neither inhibited by oxophenarsine or phenylarsenoxide (Rao and Osper, 1961).

## 4.2 Discussion of the studies on drug resistance

### 4.2.1 The development of the resistant character

Different methods were used in this thesis for the production of the resistant line of T. brucei. The basic requirements considered necessary for the method to be used were a fast production of the resistant line, reproducibility and a minimum requirement of experimental animals. The first routine tried involved exposure of trypanosomes in vitro to different concentrations of melarsen oxide; the second routine used was the continuous exposure of the trypanosomes in vivo. After exposure by either method the cells were used to infect new hosts. These two routines were abandoned because even at minimum concentrations of the drug there was a clear trypanocidal effect; another disadvantage of these routines was the uncertainty of the critical dose at which trypanosomes could not be recovered after exposure in vivo or in vitro.

Other methods have been proposed for the development of resistant trypanosomes in vivo (Schnitzer and Grunberg, 1957); the short passage method was chosen instead of the relapse method as it has been suggested that the former does not produce serum resistant organisms (Bishop, 1959). The resistant line of T. brucei (from stabilate [BEU melarsen oxide 0.52]) was produced after ten weeks with sixteen passages. The fast line developed against sodium melarsen (Rollo and Williamson, 1951) using the relapse method was produced after eighteen weeks and thirty passages.

By comparing the doses of melarsen oxide used at the beginning and at the end of the experiment a gross resistance factor of 13 fold is obtained for the melarsen oxide resistant line; an eleven fold resistance factor was observed by Rollo and Williamson (1955) for the line resistant to sodium melarsen. Therefore a comparable level of resistance has been obtained in the line of T. brucei developed in the present work.

The original parent line of T. brucei used in this work is a relatively old laboratory adapted population of trypanosomes. Similar results to the ones presented above, with respect to the time for production of the fast trypanosomes, were reported by Hawking and Walker (1966) and Walker (1964), when working with another laboratory adapted line of T. brucei. They reported that the time required for the production of the resistant line depends on the random appearance of resistant mutations in the population. This is apparently valid as the same authors reported that resistance develops very readily in freshly isolated populations of trypanosomes without previous treatment with arsenicals. Therefore it is suggested that a similar mechanism may be responsible for the development of resistance to melarsen oxide in the line of T. brucei used in this work. Further discussion on this aspect is covered in Section 4.2.4 of this Discussion.

With regard to the reference collection developed throughout the production of the resistant line it is suggested that a line produced from the stabilate [BEU melarsen oxide 0.52] could be called a strain as soon as the population of trypanosomes is characterised in relation to the resistant character. There is a clear evidence of ancestry between the new line developed and the original parent line. These suggestions are made according to the proposals for the nomenclature of salivarian trypanosomes and for the maintenance of reference collections, recommended by the World Health Organisation (1978). Although most of the work described in this thesis was done with the original parent line and the line developed from stabilate [BEU melarsen oxide 0.52] a similar approach can be taken with the intermediate stabilates produced. New strains could be developed and it could be established whether or not there is a correlation between the appearance of the resistant character or characters and the stage of exposure according to the pedigree produced (Figure 3.1).

4.2.2 The drug sensitivity test; validity of the parameter used

Trivalent arsenical compounds have been found to inhibit the oxygen consumption of T. rhodesiense and T. evansi (Fulton and Christophers, 1938; Marshall, 1948). Indeed it was later found that this is not surprising since arsenicals inhibit aerobic glycolysis, a metabolic pathway linked to the reduction of molecular oxygen in trypanosomes (Ryley, 1956).

The production of the resistant line was only possible, under the conditions described, because of the continuous monitoring of the different relapses obtained after treatment with increasing concentrations of melarsen oxide (Figure 3.2). The drug sensitivity test used for the routine analysis of the treated population of trypanosomes, was based on the sensitivity of these organisms to inhibition of the oxygen consumption when treated with different concentrations of the trivalent drug. The glycolytic sequence is the unique source of energy of these organisms (Bowman and Flynn, 1976); also the LS forms of these parasites lack cytochromes capable of oxygen reduction (Fulton and Spooner, 1959; Ryley, 1962). Therefore, the mitochondrial glycerophosphate oxidase being the only oxygen consuming system in the LS forms of these parasites (Opperdoes et al., 1976), it was assumed that whatever the mechanism of resistance it was possible to detect it as a change in sensitivity to inhibition of the oxygen consumption.

The drug sensitivity test developed, produced the results expected since it was possible to differentiate between the lines of trypanosomes with clear differences in sensitivity to the drug. However the method had one slight disadvantage in that the system did not produce linear traces of oxygen consumption at above 40% inhibition (approximately) a feature explained in terms of the trypanocidal effect of the drug in the parent line. This disadvantage of the system does not, however,

preclude the detection of the resistant character as lower inhibition of the oxygen consumption was expected with the possible resistant lines.

4.2.3 The oxygen consumption and the probit analysis

It was observed with the drug sensitivity test, that a reduction in oxygen consumption is accompanied by a reduction in the number of motile organisms present after treatment with the chemotherapeutic agent *in vitro*; thus it is assumed that the oxygen consumption of a determined population of trypanosomes is a reflection of the number of active organisms. Furthermore, the motility and the oxygen consumption decrease more quickly as the drug concentration is increased. Therefore it was decided to correlate the oxygen consumption observed with the drug concentration used. For the purposes of this analysis, the linear portions of the rates of oxygen consumption are compared in the different populations of trypanosomes. The results obtained for the residual uptake were transformed using the probit analysis, which is a linearisation of the drug response analysis (Mather, 1964). According to this author, this type of analysis is frequently used in toxicology and bioassay experiments involving similar situations to the one described above. The analysis was also used by Hawking and Walker (1966), to study the development of resistance to trivalent tryparsamide in *T. rhodesiense* and *T. brucei*.

The assumption made with respect to oxygen consumption, as a general characteristic of the population, was apparently valid as a linear response was observed when the results for the parent line are presented using the probit transformation (Figure 3.3). The probit transformation being a linearisation of the standard distribution of sensitivities to the drug in the population, it is suggested that there is an apparently homogeneous population of trypanosomes in the parent line with respect to drug sensitivity.

When the results obtained with the resistant line are treated similarly, a linear biphasic relationship is found (Figure 3.3). This type of relationship has been interpreted as evidence of a heterogenous population of trypanosomes with different degrees of sensitivity to the drug (Hawking and Walker, 1966). The plateau found at probit value 3.75 implies that only about 90% of the population is resistant to the drug; this conclusion was difficult to reach in practice with the resistant line, as the inhibition of motility was only evident at a higher concentration of the drug ($250 \times 10^{-6}$M). Therefore it is suggested that although a mixed population may exist in the new resistant line of T. brucei a low sensitivity to inhibition of the oxygen uptake may be responsible for the biphasic results obtained. This situation can be finally clarified in the future if the population is subjected to cloning and similar studies are performed on populations derived from a single organism.

4.2.4 Further studies on the development of the resistant character using Warburg manometry

The routine drug sensitivity test performed with the oxygen electrode allowed the analysis of populations of trypanosomes at low concentrations of organisms with good rates of oxygen uptake; this probe detects very small changes in the oxygen tension in solution. However in the metabolic section of this thesis, the experiments were performed using constant volume Warburg manometry; this technique allows the handling of higher concentrations of organisms for longer periods of time. Therefore in order to have a comparable body of evidence, some data was obtained on the development of the resistant character using the latter technique. A comment should be made on the meaning of the $I_{50}$ values obtained ($I_{50}$ = drug concentration to produce 50% inhibition). With the oxygen electrode the inhibition is considered on the initial (linear) rates of

uptake; with the Warburg manometric system the inhibition considered is the one produced over a period of 30 minutes incubation with the drug. The reason for this long period of incubation is to maximise the production/consumption of metabolites under the conditions used (see Figure 3.8). It was found that the $I_{50}$ values obtained were higher with the manometric system; this difference is apparently due to the presence of serum albumin in the buffer. It is suggested that the protein may decrease the concentration of free drug by unspecific binding. Organisms from the first relapse, after infection of rodent hosts with stabilates, were used to study the development of the resistant character (see Figure 3.4). This precaution was taken as the stability of the resistant character was only studied in the resistant line from stabilate [BEU melarsen oxide 0.52] and no information is available on the other intermediate lines (see Section 4.2.5). The pattern of inhibition found corroborated the results obtained with the drug sensitivity test showing great differences between the parent line and the resistant line (from stabilate [BEU melarsen oxide 0.52]). By looking at the results obtained (Figure 3.4) it may be suggested that the patterns of inhibition are similar in the populations exposed to 0.06 to 0.12 mg/kg and the parent line. Comparing the levels of inhibition in the $O_2$ consumption at $10^{-5}$M melarsen oxide:

| Stabilates | % Inhibition at $10^{-5}$M melarsen oxide |
|---|---|
| Parent | 90 |
| [BEU melarsen oxide 0.06-0.0.12] | 90 |
| [BEU melarsen oxide 0.18] | 78 |
| [BEU melarsen oxide 0.32] | 62 |
| [BEU melarsen oxide 0.52] | 15 |

It appears that there are intermediate levels of sensitivity in the different lines studied. The results obtained are simiilar to those reported by Hawking and Walker (1966) for the development of a tryparsamide resistant line from a laboratory adapted parent line of

T. brucei. Because of the similar patterns of sensitivity found with populations exposed to a low concentration of the drug and the parent line it is concluded that the resistant character does not preexist in the parent line; furthermore the final resistant character is apparently developed in stages after the selection of less sensitive organisms, thus different degrees of heterogeneity with respect to the sensitivity to the drug may exist in the different stabilates used. It is also suggested that the melarsen oxide resistant line produced in this thesis may have been developed after the appearance of one or more spontaneous mutations which are selected under the pressure of the drug.

4.2.5 The stability of the resistant character

It has been previously found that resistance, when fully developed, is a relatively stable character even in the absence of the drug to which the resistance was developed (Murgatroyd and Yorke, 1937; Bishop, 1959). Stability may be assessed either by serial passage in rats or by passage through the insect vector (Gray and Roberts, 1968); the latter method of testing the stability of resistance was discarded as the parent line used in the experiments is a laboratory adapted line.

After testing the melarsen oxide sensitivity of the resistant line in the absence of drug pressure and after serial passage in rats, it was found that the character was stable up to the 13th passage (Figure 3.5). The apparent stepwise manner in which the resistant line was obtained (as discussed in the last section), and the stability of the character under the conditions used may suggest that the new feature is well established in the genetic pool of the population of trypanosomes. The position may not be the same for the other lines produced at lower resistance factor levels. Similar experiments (Hawking and Walker, 1966) suggest that a stable character is only found at high resistance factor

levels. It should be mentioned that although it was found that the resistant character was stable in terms of sensitivity to the drug, all the experiments were performed with organisms from the first relapse after infection from stabilates; the reason was that no information was available on the stability of any other possible character in the newly developed line of T. brucei e.g. metabolic differences or enzymic differences.

The next question to answer was about the stability of the new line in vivo under pressure of the drug. The results obtained (Figure 3.6) showed that the resistant line was able to kill the rodent host despite drug treatment, whereas the parent line was completely cleared from the circulation after treatment with the drug. However under treatment with the drug in vivo, there was a delay in the development of the parasitaemia in the resistant line with a subsequent delay in the death of the animal. Two alternatives are put forward to explain this observation. First that there is a mixed population of trypanosomes as suggested in Section 4.2.3. In spite of the high level of organisms supposed to be resistant in the population (about 90%) there was a considerable delay in the development of the parasitaemia. Therefore the second suggestion is that the resistant line itself remains partially sensitive to the drug and therefore a delay in the development of the parasitaemia is observed. This proposition is difficult to substantiate as the work has been done with a possible heterogeneous population of resistant parasites without previous cloning.

The two alternatives proposed above have not taken into consideration the possibility of direct participation of the host in the control of the infection. The immune system of different hosts infected with trypanosomes has been studied and noticeable increases in the activities of certain mechanisms have been observed (Herbert and Parrat, 1979).

Therefore the combined effect of partial sensitivity to the drug and to the immune system of the host may explain the delay in the development of the parasitaemia in the resistant line after treatment with the drug.

4.2.6 Testing different hypotheses for the development of resistance

The main general mechanisms which might be responsible for the development of resistance in trypanosomes were discussed in the Introduction, and most of the evidence gathered on the development of resistance to the arsenicals, including those of the melaminyl series, has pointed towards two main alternatives; first the development of a metabolic bypass, and second a reduced uptake of the drug. A new melarsen oxide resistant line of T. brucei was used in this work therefore it was necessary not only to cover the two alternatives mentioned above but also the possibility of modification of enzyme targets for the drug as a cause of the development of resistance.

The general strategy was as follows: the metabolism of carbohydrate by parent and resistant lines was first compared, as much information was available on this particular topic for the parent line. Any information obtained from this primary analysis could be used as a general starting point from which further suggestions may be formulated.

Second a comparison was made in terms of the kinetic parameters and drug sensitivities of two enzymes, PK and PGK. One of these (PK) has already been pin-pointed as a focal point for the action of melaminyl arsenicals. Third a closer look was taken at the uptake of the drug by parent and resistant lines in order to characterise the mechanism of transport in both populations of trypanosomes.

4.2.7 General carbohydrate metabolism in parent and resistant lines

From the preliminary metabolic experiments performed (Table 3.2) it is suggested that the parent line of T. brucei (LS form) used in this work has the same metabolic characteristics as other trypanosomes

of the subgenus Trypanozoom (Ryley, 1956; Grant and Fulton, 1957; Brohn and Clarkson, 1978). It was found that the aerobic consumption of one mole of glucose produced approximately two moles of pyruvate (Table 3.3); and that the consumption/production of these metabolites proceeds at an extremely high rate. The results of these preliminary experiments were not the same when the resistant line was investigated, as a lower rate of pyruvate production was observed and therefore a lower yield of pyruvate per glucose was found. Although traces of glycerol are found in both parent and resistant lines, the amounts produced did not increase as a function of time. Thus it is concluded that the appearance of this metabolite is of minor importance as the traces of glycerol may be due to a transient anaerobic state during the experimental handling of the trypanosomes.

When the general carbon balance of both organisms was studied using constant volume Warburg manometry (Table 3.3), the same metabolic features were found with parent and resistant lines as described in the preliminary carbon balance experiment. The most important aspect is that the lower pyruvate production, when compared to the glucose and oxygen consumption, is corroborated in the resistant line (see Table 3.3). Another difference found with respect to the parent line was the production of $CO_2$ with a RQ of about 0.1 in the resistant line. Different alternatives can be put forward to explain the results obtained:

a. Production of $CO_2$ metabolism of glucose via the hexose monophosphate shunt.

b. Production of $CO_2$ by decarboxylation of pyruvate with the subsequent production of another metabolite.

c. Production of $CO_2$ in the tricarboxylic acid cycle (TCA).

As for the alternative "a", experiments using specifically labelled $^{14}C$ glucose have suggested that the pathway is operational in different lines of T. cruzi (Mancilla and Naquira, 1964). Similar studies in

some members of the trypanozoon subgenus have shown that the hexose monophosphate pathway does not operate to a significant extent, as less than one per cent of the carbon from glucose is found as $CO_2$ (Bowman, 1974). Furthermore the apparent absence of 6-phosphogluconate dehydrogenase, (Flynn, 1971) shows this pathway very unlikely as the source of carbon dioxide. On the other hand if the pathway were operational in the resistant line this would not explain alone the lower pyruvate/glucose ratio found.

The metabolic characteristics of the resistant line of T. brucei are in fact not different from those of LS forms of other members of the Brucei group, in which some $CO_2$ is produced as a metabolic end product of glucose metabolism (Ryley, 1956; Flynn and Bowman, 1973). It is therefore suggested that in the resistant line, decarboxylation of pyruvate may occur. However no definitive conclusion can be reached at this stage with respect to the further production of $CO_2$ or about the nature of the other metabolites involved, as succinate and acetate may be present as the other final products of glucose metabolism in the blood stream forms of these parasites. Therefore alternative "b" can not be discarded at this stage. With regard to alternative "c", it has been suggested that the TCA cycle is not fully operative in the LS forms of the parasite (Bowman and Flynn, 1976); instead the possibility of succinate formation, involving $CO_2$ fixation with reversal of some of the enzymes of the TCA cycle, has been suggested (Klein et al., 1975). It is clear that in the hypothetical case of alternative "c" this will immediately include "b".

The possibility of a metabolic modification as a result of the development of resistance to arsenicals has been studied in the past;

earlier evidence of Reiner et al. (1932) when comparing the respiration rate of normal and neoarspenamine resistant T. equiperdum did not suggest any major modification in the resistant line. The same conclusion was reached by Harvey (1949) when studying the metabolism of carbohydrates in an oxophenarsine resistant line of T. hippicum.

The results presented in this thesis suggest the possibility of a minor modification on the metabolism of the melarsen oxide resistant line when studied without the influence of the drug. Modifications, in this context, were also found in a tryparsamide resistant line of T. gambiense (von Brand et al., 1953); this line showed lower rates of glucose consumption and pyruvate production. It was in general suggested that the characteristic observed were not directly related to the development of resistance; nonetheless it was inferred that an alternative pathway was playing a more important role in the resistant line. A similar type of situation is observed in this work, as the metabolism of the resistant line in the presence of the drug suggested the possibility of an alternative pathway for the fate of pyruvate (see Section 4.2.8).

A distinct change in the metabolism was found in a sodium melarsen resistant line of T. rhodesiense (Williamson, 1953a). Unlike the parent line, the resistant line was unable to utilise glycerol pyruvate, glutamate or succinate but showed increased ability to use citrate and lactate. Although the changes were quite pronounced it is difficult to rationalise the differences in terms of the development of resistance. Further studies on the effect of a variety of metabolic inhibitors on resistant T. rhodesiense in vitro suggested that although metabolic modifications may have occurred these were not directly related to the development of resistance (Williamson, 1959a).

Metabolic changes have therefore been observed in the past with other organic arsenicals. Indeed it is reported in this thesis that the melarsen oxide resistant line showed apparent changes in the metabolite production/consumption when compared with the parent line. Although this minor modification could suggest a less resistant secondary pathway to the drug, there is not a clear relationship in terms of the high levels of resistance observed in the trypanosome. A further analysis is made however in the Section 4.2.8 on the general metabolism of the parent and the resistant lines under the influence of melarsen oxide.

4.2.8 The effect of melarsen oxide on the metabolism of parent and resistant lines

As reported in Section 4.2.2 the inhibition of oxygen uptake was used to differentiate parent and resistant lines of T. brucei. However when the same parameter was compared in water lysates from both lines of trypanosome (Figure 3.10) it was found that there was no difference; therefore it is suggested that common sites of inhibition exist in both lines of the parasite which become available in the resistant line on disruption of the cell membrane. Alternatives for the sites of inhibition are discussed below. With respect to the different patterns of inhibition found in whole cells and the similar pattern found in water lysates, it is suggested that a permeability barrier for the drug may be involved in the resistant line. The nature of this permeability barrier is dealt with in more detail in the drug transport section (4.2.13) of this Discussion.

As mentioned above common intracellular sites of inhibition are proposed for both lines of the parasite. Comparison of the inhibition of metabolite production/consumption in parent and resistant line organisms by melarsen oxide (Section 3.3.4) suggested that pyruvate

production could be a site of action; howefer the $I_{25}$ values were considered for the resistant line and $I_{50}$ values for the parent line. The observation that a higher extracellular concentration is needed to produce inhibition in the resistant line substantiates the possibility of an acquired permeability barrier for the drug. In relation to the metabolite inhibition, it was found that the $CO_2$ production in the resistant line is extremely sensitive to inhibition by melarsen oxide as no $CO_2$ was detected at concentration above 90 x $10^{-6}$M drug. Oxygen and glucose consumption, and pyruvate production are only inhibited between 25-30%, at the same concentration of drug. Therefore it seems that the further metabolism of pyruvate (assuming that this is the source of $CO_2$) is apparently more sensitive to melarsen oxide inhibition.

It has been proposed since the work of Krebs (1933a,b) that arsenite causes the accumulation of α-ketoacids in kidney preparations. These experiments and those of Peters (1955) and Peters et al. (1946) confirmed that pyruvate oxidase, from mammalian tissues and from pigeon brain, was the focal point of action of arsenicals; furthermore it has been shown from studies on a pleomorphic line of T. rhodesiense that pyruvate oxidase (from SS forms) is also sensitive to trivalent organic arsenicals (Flynn and Bowman, 1974). It was also reported by the latter authors that although pyruvate decarboxylation is sensitive to the arsenical drugs, the main site of lethal action is PK.

The pyruvate/glucose and pyruvate/oxygen ratios were apparently constant, even at high concentration of the drug, in the resistant line when compared with the parent line (Table 3.4) which showed a reduction in the pyruvate/glucose ratio with increasing drug concentrations. Also if the inhibition of $CO_2$ production is assumed to come from the decarboxylation of pyruvate it can be inferred, that although there is

inhibition in the metabolism of the resistant line another metabolic route for pyruvate should exist; furthermore this hypothetical metabolic route plays a more important role in the resistant line when the drug is present. As discussed in the last section, Williamson (1953a) described a sodium melarsen resistant line of T. rhodesiense which developed lactate dehydrogenase activity which was not present in the original parent line. However, no information was obtained with regard to this characteristic in the resistant line used in this work.

Negative RQ values were observed when studying $CO_2$ production in the resistant line at high extracellular concentrations of melarsen oxide (Figure 3.13). This observation and that of Klein et al. (1975) who reported the presence of $CO_2$ fixing enzymes in T. brucei could be used to infer the posssibility of $CO_2$ fixation in the resistant line when exposed to the action of the drug.

In order to clarify this minor metabolic difference found in the resistant line, the suggestions proposed by Bowman and Flynn (1976); about the utilisation of radioactive precursors to estimate the specific activity of individual carbon atoms in metabolic intermediates and end products and the identification of the enzymes involved in the pathways proposed, should be followed.

With regard to the $CO_2$ production in the resistant line, there is no apparent evidence of pleomorphism in the population of trypanosomes as shown in Figure 2.2; therefore a viable alternative is the possibility of an intermediate form of the parasite capable of oxidative decarboxylation which appears to be sensitive to melarsen oxide inhibition in the resistant line. This possibility is the most likely alternative, nevertheless this can only be inferred.

Finally the metabolic differences observed in the parent line, with and without melarsen oxide in the incubation medium, have only

been studied and analysed to a limited extent because as already mentioned, it was found that the main suggestion to explain the development of the resistant character was in terms of a permeability barrier for the drug.

4.2.9 The glycosome sensitivity to melarsen oxide and the thiol content of parent and resistant lines

It has been suggested that several members of the Order kinetoplastida have the particulate organelle called the glycosome (Taylor et al., 1979), and that this particle is surrounded by a limiting membrane which allows a certain level of compartmentation (Visser and Opperdoes, 1980). The glycosome is therefore potentially important in the development of resistance. However when the activity of the whole organelle from parent or resistant lines was studied under the influence of melarsen oxide, no differences were found in the inhibition patterns (Figure 3.14). Although the multienzyme activity of the glycosome is inhibited to the same extent in both parent and resistant lines, this does not rule out the possibility of different enzymes in the glycosome having different kinetic properties or being susceptible to different levels of inhibition by the drug.

Another feature which has been suggested as an explanation of the development of resistance is the production of an excess of thiol groups in the resistant line which will detoxify the drug. Although this hypothesis has been tested before (Hawking, 1938; Harvey, 1948) and apparent differences were not found, it was necessary to check this feature in the new resistant line of T. brucei. The precaution was taken this time to estimate both available and total thiol after folding of the protein (Figure 3.15). Again no differences were found for either thiol type between parent and resistant lines. Therefore this hypothesis can be discarded as a possible mechanism for the development of resistance.

4.2.10 The enzymological studies on parent on resistant lines

From the metabolic section of this Discussion it has been suggested that although a minor modification exists in the resistant line, the development of resistance is probably associated with a difference in drug permeability between the lines of parasite. However it must be remembered that the mechanism of action of organic arsenicals involves a preliminary binding of the drug to the parasite, followed by reaction with specific enzymes in the cell (Williamson, 1970). Therefore modification at the level of target enzymes is not completely ruled out from the metabolic results obtained. Indeed the possibility has already been described for a pyrimethamine resistant line of Plasmodium berghei which has a modified dihydrofolate reductase activity, ten fold greater than in the parent line (Ferone, 1970). Modification of the kinetic parameters of the enzyme in the resistant line were also reported (Pinder, 1971).

A more detailed study of the general characteristics of some possible target enzymes was therefore carried out. In the first place PK was investigated as this enzyme has been reported to be the main target of organic arsenicals in the parent line (Flynn and Bowman, 1974). Other potential targets are difficult to pinpoint as a modification may arise in any of the enzymes sensitive to melarsen oxide. The other enzyme chosen for investigation was PGK as it functions similarly to PK i.e. it is involved in phosphorylation at the substrate level. The ATP produced by these enzymes is considered to be the only source of energy available to the parasites (Bowman and Flynn, 1976).

Three alternative enzymological explanations for the development of resistance are: 1) modification of the kinetic parameters of the enzymes for their substrates, 2) increased specific activity to overcome inhibition by the drug and 3) a lowered sensitivity to the drug in the

resistant line enzymes. These features were investigated in semicrude preparations (see Figure 3.16) of the two enzymes in order to avoid any modifications due to a more stringent purification procedure.

a) Pyruvate kinase

Study of the kinetic parameters of PK corroborated previously published data (Flynn and Bowman, 1980) (Table 3.7). The enzyme is homotropically allosteric with respect to the substrate PEP. After addition of the heterotropic activator, FDP, there was no modification of the $V_{max}$ of the enzyme allowing classification as a "k" enzyme (Monod et al., 1965). The same kinetic parameters are found for parent and resistant line enzymes with regard to the $S_{50}$ and $V_{max}$ values for PEP in the presence and absence of FDP. The only minor difference observed was the Hill coefficient value obtained for PEP binding in the absence of FDP in the resistant line. However the age of the enzyme preparation and the use of different batches of lyophilised material can produce some variation in this parameter. Furthermore it has been suggested (Flynn and Bowman, 1980) that the enzyme is fully activated by FDP in vivo under conditions of high glycolytic activity. For these reasons no significance can be attributed to this apparent variation between parent and resistant line PK. The response of PK with respect to ADP was found to be of the simple Michaelis-Menten type and again $K_m$ and $V_{max}$ values were similar in both lines. Thus, it was concluded that the main target of organic arsenicals is apparently similar in parent and resistant lines as far as kinetic properties are concerned.

Finally it should be mentioned that the activity of the enzymes was considered in terms of rate/mg protein for all enzymological studies presented. The analogous $V_{max}$ values are therefore evidence of similar specific activities of the enzymes from parent and resistant lines.

b) Phosphoglycerate kinase

This enzyme was also investigated because of its involvement in energy transduction and because PGK has been poorly studied in trypanosomes. Discussion at this point will be limited to the possible involvement of this enzyme in the development of resistance. The general enzymic properties are dealt with in more detail in Section 4.1

Estimation of the kinetic parameters of the enzyme from both sources with the technique already described (Table 3.8) showed that the respective Michaelis-Menten constants and maximal velocities for both substrates, G3P and ATP, were the same for parent and resistant line enzymes.

Again the maximal velocities are an indication of the specific activities of the enzymes from the different sources. Therefore, as found with PK, the enzyme in the two lines is apparently the same.

4.2.11 The effect of melarsen oxide on pyruvate kinase and phosphoglycerate kinase from parent and resistant lines

Exclusion of modification of the kinetic parameters and the possible development of increased concentration of the enzymes in the resistant line does not rule out the third alternative of differential inhibition by melarsen oxide between the enzymes from parent and resistant lines. As suggested by Sevag (1955) and Schueler (1947) conformational changes of the structures involved in the binding of drugs may alter the affinities of the latter for their targets. However when the patterns of inhibition of the two enzymes are studied (PK; Figure 3.20; PGK; Figure 3.28) no significant changes are apparent under the experimental conditions used. As mentioned previously, semicrude fractions were used as the source of the enzymes without major purification (2-3 fold). Therefore the concentrations of drug presented as the $I_{50}$ values are probably overestimates as there are other proteins present with available

thiol groups that may react with melarsen oxide and hence produce an artificial reduction in the concentration of the free drug able to react with the enzymes under study.

To summarise, investigation of some drug targets in parent and resistant lines was carried out to substantiate the suggestion made from metabolic studies; no metabolic differences were found to be responsible for the development of resistance and from the results obtained the possibility of permeability differences for the drug was inferred. The alternative of modified targets was thought worthy of clarification prior to drug uptake studies and the lack of enzymological differences found between the lines of the parasite reinforces the alternative of differential uptake as the major feature of the phenomenon of resistance. This topic is the subject of the next section.

4.2.12 The drug transport studies and the setting of the experimental conditions

For the study of melarsen oxide transport in T. brucei several parameters were controlled in order to ensure that, under the conditions used, the uptake mechanism was a real characteristic of the cell population. These precautions had to be taken with this drug due to its properties as a strong metabolic inhibitor.

The incubation of large numbers of trypanosomes ensured that measurable amounts of the drug were taken up by the cells. The temperature ($21^{\circ}C$) (Figure 3.36) and the time of incubation (60 seconds) (Table 3.9) with different concentrations of the drug were selected in such a way that there was 100% motility in the cell population at the end of the incubation.

Another requirement for this type of study is the availability of a specific assay system for the transported substance. This requirement was fulfilled by using an assay system specific for the estimation of

arsenic in the drug (US Pharmacopeia 1975).The sensitivity of the system used (Section 3.6.1) allowed accurate measurement of the drug down to the microgram level.

Due to the fast reaction of trivalent arsenicals it was concluded that the mechanism of translocation should be rapid to allow for the fast action of the drug at the intracellular site of action. Therefore a rapid exposure system was used in order to ensure good estimates of the initial rates of transport (Figure 3.34). The silicone sandwich technique, as described by Damper and Patton (1976b) was used for this purpose. One minor disadvantage of this technique is the extracellular fluid pulled by the cells through the silicone layer when centrifugation is performed (see Materials and Methods). This problem was solved by calculating the fluid volume involved using an impermeable extracellular marker ($^{14}C$ inulin) and correcting for the amount of drug associated with this volume (Table 3.10).

In a preliminary experiment on the distribution of melarsen oxide it was found that most of the drug was associated with the acid insoluble pellet of protein (Figure 3.35). It is therefore suggested that a relatively stable interaction exists between the drug and the denatured protein. The nature of the stability of the drug-protein interaction, in terms of the arsenic-thiol interaction, is covered in more detail in Section 4.1.4.

4.2.13 The kinetics of the transport of melarsen oxide in parent and resistant lines

As mentioned in the Introduction, some information available on the transport of arsenicals into trypanosomes indicates a reduced uptake in the resistant lines investigated. The evidence obtained by Yorke et al. (1931) and Hawking (1938) was gathered using a biological assay for the drug, although in the latter case a chemical estimation was also

used. The experiments performed by these authors were mostly on laboratory produced resistant trypanosomes. Eagle and Magnuson (1944) found similar results with a spontaneously developed arsenical-resistant line of T. equiperdum. Since then these results and others have been used to explain the development of resistance in trypanosomes. Most of the data have been obtained from relatively long periods of exposure of the organisms to the active agents. In other words there is no definitive evidence available on the mechanism by which the uptake of the drug is modified or suppressed. Therefore the approach followed in this work was to study the kinetics of drug transport in parent and resistant lines in the first place.

The experiments performed showed (Figure 3.39) that the initial rate of transport, as a function of the drug concentration, approached a maximum velocity in the parent line which suggests the presence of a saturable component for the transport of the drug. Similar results to those presented here were observed for the trasnport of diamidines in T. brucei (Damper and Patton, 1976a) in which the saturation kinetics for transport mechanisms were interpreted as evidence of a carrier mediated mechanism.

With regard to the kinetic parameters of the transport system in the parent line it should be mentioned that the scatter found in the experimental values precludes the use of linear transformations of the data to estimate the kinetic constants. Instead non-linear regressions were applied in order to calculate the parameter. Two computer programs were used, one for the Michaelis-Menten equation (Atkins and Gardner, 1977) and the other for the estimation of the parameters of the Hill equation (Atkins, 1973). The second program was found to give a better fit to the data obtained. Therefore the flux of melarsen oxide into the parasite did not show a typical Michaelis-Menten pattern, suggesting a

more complicated mechanism for the transport of melarsen oxide in the parent line. The kinetic parameters obtained are interpreted as the maximal velocity of the transport of the drug ($V_{max}$) and the $S_{50} = K_t$ or substrate concentration at which the rate of transport of the drug is half maximum. The meaning of the Hill coefficient is discussed below.

The possibility of complex carrier mediated mechanisms has already been reported by Goldman (1973). It was shown by the same author that typical Michaelis-Menten patterns for undirectional fluxes are sometimes difficult to demonstrate experimentally. The observation that the Hill equation (Hill, 1910) was the closer mathematical model for the data obtained for the transport of melarsen oxide in the parent line, may suggest cooperative binding of the drug. If this is so then the Hill coefficient (n value) observed in the carrier mediated system will represent the cooperative index for the drug when binding to its receptors. By analogy with an enzymic reaction, it may be suggested that the transport system for melarsen oxide is activated by the drug in a homotropic fashion.

Allosteric effects and conformational changes, as in enzymic reactions, have been suggested as unsatisfactory models to explain the movement of some substrates across membranes (Selwyn et al., 1977). The view of less satisfactory models is based on the distances involved in membrane transport. However as suggested by the same author, a combination of allosteric protein conformation changes with possible pores in the membrane are postulated to overcome the problem. The possibility of allosteric transport mechanisms has also been discussed by Lieb and Stein (1972). They concluded that shuttles or pores could not account for the kinetics of glucose transport in red cells. Therefore it was proposed, not only for this carrier but also for the ($Na^+/K^+$) dependant adenosine triphosphatase (Stein et al., 1973),

a model of transport based on the allosteric transformations of haemoglobin.

In conclusion the transport mechanism for melarsen oxide was found to be complex in the nature of the interaction between the drug and its receptors. Further experiments should be performed on this particular topic, if possible with a more accurate system for estimation of the drug, in order to rationalise the characteristics observed for this transport mechanism.

With regard to the transport of melarsen oxide in the resistant line only a residual and apparently linear rate of transport is observed, as a function of the drug concentration (Figure 3.39). The first order kinetics observed for the transport of the drug is suggested as evidence of a simple diffusion system (Neame and Richards, 1972). Therefore it is concluded that the resistant line of T. brucei developed in this work has lost the carrier mediated transport, responsible for most of the transport of the drug, as a result of the development of the resistant character. Instead a simple diffusion is alone responsible for the uptake of the drug in the resistant line. Some evidence to support this is found in Section 3.3. It was shown that the metabolism of carbohydrates is only inhibited at considerably higher extracellular concentrations of the active agent in the resistant line. It is suggested that at high levels of extracellular drug some diffusion into the parasite occurs simply because of the gradient of concentration between the extracellular and intracellular compartments. Considering that the mechanism of action of arsenicals involves two stages, binding to the cell and then lethal action (Williamson, 1970), it is logical to assume that the reduced effect found on metabolism is the result of lower absorption of the drug.

The results obtained in this work are in agreement with the cross resistance pattern of different lines of trypanosomes fast to different drugs (Williamson and Rollo, 1959). It was then suggested that

stereospecific changes at the level of the binding of the drug are associated with the development of resistance. Similarly the results presented in this thesis suggest that this modification has occurred to the extent that the carrier mediated system is no longer able to recognise the drug. The possibility of complete physical deletion of the carrier is regarded as untenable, as it is difficult to imagine from the evolutionary point of view, how an organism with a high degree of specialisation has developed a transport mechanism specific for the uptake of the drug. Instead it is suggested that the drug normally shares the transport mechanism of some other metabolite which presumably is still required in the resistant line. No experiments were performed in this work to establish the nature of this metabolite; however it is inferred that the latter must have some structural relationship with melarsen oxide.

An example of the kind of approach that could be followed is to compare the transport mechanism of a structurally related metabolite and also to study the mechanism of inhibition by the drug and in this way it could be determined whether or not the same transport system is being shared. A possible candidate for this could be the transport of purines in T. brucei, metabolites which are not synthesised by the parasite but salvaged from the host bloodstream (Gutteridge and Rogerson, 1979). The purines are nitroheterocyclic compounds which may have some resemblance with the triazine ring of melarsen oxide.

Regarding the developments of resistance; the other uptake mechanism so far investigated is that of pentamidine in T. brucei (Damper and Patton, 1976b). These authors found that the resistant lines studied developed a transport mechanism for the drug, which was less efficient in terms of lower $V_{max}$ and higher $K_m$ values. They interpreted these kinetic parameters as indicators of a reduced number of binding sites with also lower affinity for the drug in the resistant

lines. Similar results to those presented here with a reduced uptake as a result of the development of resistance have also been reported for a chloroquine fast line of Plasmodium berghei (Macomber et al., 1966).

Finally, an estimate can be obtained for the actual intracellular concentration of melarsen oxide at initial velocities approaching saturation of the drug carrier in the parent line (Figure 3.39).

| | |
|---|---|
| -Trypanosomal suspension used in uptake experiments | 45 µl Total cell volume/ml suspension . (1/22 v/v packed cell volume in buffer) |
| -Volume of suspension used in uptake experiments | 0.5 ml |
| -Approximate amount of melarsen oxide found in the pellet at $80 \times 10^{-6}$ M extracellular concentration | $13.3 \times 10^{-9}$ moles |

Therefore:

$$\text{Intracellular concentration of melarsen oxide} = \frac{13.3\ 10^{-9}\ \text{moles}}{(45\ \mu\text{l} \times 0.5)} = 0.59\ 10^{-3}\ \text{M}$$

The figure obtained for the intracellular drug concentration is only an approximation to the real value. It is in fact an underestimation, as the packed cell volume was used for the calculations. In spite of this observation the transport of melarsen oxide is considered to be concentrative as the extracellular concentrations used during the uptake experiments were all in the micromolar range. The same conclusion is obtained from the studies of Hawking (1938) on the transport of other different aresenicals.

Concentrative transfer has been observed in different tissues specially with amino acids (Johnstone and Scholefield, 1965; Neame, 1968; Wilbrant and Rosenberg, 1961). Indeed it is suggested as a general characteristic of some carrier mediated processes (Goldman, 1973; Stein, 1967).

4.2.14 The influence of temperature on the transport of melarsen oxide in parent and resistant lines

The influence of temperature on a transport mechanism provides valuable information on the nature of the mechanism itself (Goldman, 1973). The influence of this parameter on the rate of uptake of melarsen oxide by parent and resistant line organisms is used to substantiate the results already obtained in the concentration dependance experiments of the last section.

A transport mechanism with a high $Q_{10}$ was observed in the parent line, compared to a low $Q_{10}$ transport mechanism at very low rates of transport of the drug, in the resistant line (Figures 3.36 and 3.37)

The temperature dependance of the uptake of melarsen oxide in the parent line suggests a carrier mediated process of transport (Kaback, 1968; Koch, 1964; Goldman,1973). The molecular implication of temperature dependant transport is not completely clear; however it is suggested that this temperature dependance is connected with the structure of the phospholipid matrix of the membrane (Kaback, 1968), which in turn defines the fluidity characteristics (Harrison and Lunt, 1975). It has also been reported that membrane fluidity is directly correlated to the permeability properties of the membrane (Chapman and Dodd, 1971).

The low $Q_{10}$ transport observed in the resistant line may be interpreted in terms of simple diffusion. It has been suggested that in general the temperature sensitivity of a simple diffusion system is not as great as that of a carrier mediated process (Goldman ,1973).

4.2.15 The thermodynamic characteristics of the transport system in parent and resistant lines

Assuming that the transition state of a drug entering a membrane is describable from data on the temperature sensitivity of the rate of

transport the Arrhenius equation may be used to define the thermodynamic parameters of the mechanism of transport of the drug. This type of analysis has been performed in studies on the transport phenomenon of different permeants in various cell systems (Stein, 1967). Indeed the same approach was also used to calculate some thermodynamic parameters for the transport of pentamidine in T. brucei (Damper and Patton, 1976b).

From the data obtained on the temperature dependance of the transport of melarsen oxide (Figure 3.38), values were calculated for Ea; $\Delta S^{\ddagger}$; $\Delta H^{\ddagger}$ and $\Delta G^{\ddagger}$ for the transition state of the drug uptake in parent and resistant lines. A comment however should be made before going into further interpretation: as the rate of transport at low temperatures in the parent line approached the rate of transport in the resistant line (Figure 3.37), it is assumed that the low $Q_{10}$ transport is also present in the parent line. Therefore for the thermodynamic calculations the rate of transport in the parent line has been corrected for the low $Q_{10}$ values found in the resistant line at the different temperatures studied. With regard to the thermodynamic parameters obtained, the high Ea value found in the parent line is suggested as further evidence for the presence of a carrier mediated system. The low Ea value found in the resistant line is characteristic of a simple diffusion system. Similarly the temperature dependency of amino acid transport in bacteria has been used to substantiate the possibility of a carrier mediated system in these organisms (Oxender, 1972). As found in this work for melarsen oxide, the amino acid transport in bacteria was minimal at low temperatures.

The other two parameters $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are difficult to interpret in biological terms. However by analogy with an enzymic reaction (Cornish-Bowden, 1979), $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ of the transport mechanism of melarsen oxide may provide some information about the nature of the transition state

and hence about the reaction mechanism. A large $\Delta H^{\ddagger}$ indicates that a large amount of "stretching and squeezing" of chemical bonds is necessary for the formation of the transition state. $\Delta S^{\ddagger}$ gives a measure of the inherent probability of the transition state.

With regard to the negative values of $\Delta G^{\ddagger}$ it is suggested that the formation of the transition state is favoured in both transport processes. However further thermodynamic considerations are of limited value as other characteristics with respect to the structure of the drug carrier, the drug itself and the kinetics of the transport system play an important role in the description of the transport mechanisms.

4.2.16 The uptake of sodium melarsen in parent and resistant lines

It was found experimentally that pentavalent sodium melarsen was non-trypanocidal in vitro. The results of experiments described in this thesis, on the uptake of the pentavalent analogue of melarsen oxide, also show that the drug is not absorbed by the trypanosomes under the experimental conditions used (Section 3.6.10). A similar conclusion was reached by Hawking (1937) when working with the trivalent and pentavalent forms of tryparsamide. Therefore the suggestion that reduction of the pentavalent drug is required prior to penetration into the parasite is corroborated in this work as there was no apparent uptake of pentavalent sodium melarsen. Possibly reduction of the drug may occur on contact with host erythrocytes (Lourie et al., 1935). Supporting evidence for extra trypanosomal reduction can also be found in the work of Rollo et al. (1949), where it was found that immediately after injection with melarsen oxide the trypanocidal titres of rabbit serum reached maximal values, but that about 6 hours were needed to reach the same levels when sodium melarsen was used.

With regard to the mechanism of transport of melarsen oxide, it is inferred from the lack of transport of the pentavalent analogue that the

uptake of the trivalent arsenical may involve the binding of thiol groups at the site of the carrier for the drug in the parasites. Indeed thiol involvement is a characteristic of some carrier mediated systems (Goldman et al., 1968; van Stevenick et al., 1955). This feature is concluded from the inhibition produced by sulphydryl reagents on different carrier mediated mechanisms, including the inhibition of glucose transport in T. brucei by para-hydroxymercuribenzoate (Gruenberg et al., 1978).

Further experimental data on the effects of these antagonists, on the uptake of the trivalent analogue in vitro, should be obtained to substantiate the hypothesis of thiol involvement at the site of the carrier.

4.2.17 The effect of a metabolic inhibitor on the transport of melarsen oxide

As reported in the Introduction LS forms of T. brucei have an absolute dependance on the ATP generated from glycolysis for metabolic needs. Furthermore, iodoacetate (IAA), a classical metabolic inhibitor (Lehninger, 1975) has been reported to inhibit the metabolism of members of the Brucei group (Williamson, 1959b). When the effect of IAA was studied on the transport of melarsen oxide in the parent line (Figure 3.44), it was found that the inhibition of the transport depends on the concentration of the inhibitor. Therefore it may be suggested that the transport of melarsen oxide is apparently energy dependant under the experimental conditions used as the trypanosomes were 100% mobile after incubation with IAA for 30 seconds.

This suggestion does not rule out the possibility of thiol interaction with IAA at the membrane level. As has been discussed in Section 4.2.16 the binding of melaminyl arsenicals may involve interaction with available -SH groups. Therefore the effect of IAA may have some inhibitory component

on the transport of the drug by the possible reaction with the -SH groups at the site of uptake.

4.2.18 The effect of melamine on the transport of melarsen oxide

It has already been reported that melamine interferes with the action of melaminyl arsenical in vivo and that it occurs at the level of binding of the drug to the parasite (Williamson, 1959c).

The results presented in this thesis show that the inhibition produced by melamine on the transport of melarsen oxide is directly related to the concentration of the inhibitor (Figure 3.41). Further investigation on the binding of melarsen oxide by the parasite, in the presence of malamine, suggests that the inhibition of the transport was mainly due to competition with the drug at the site of uptake (Figure 3.42). It is also inferred from the nature of the competition that the transport of melarsen oxide is specific for the melaminyl residue of the drug, as the inhibition is sharing part of the binding site with a major competitive component. It is also inferred from these experiments that the malminyl ring in melarsen oxide is required for the recognition of the drug at the binding site. As reported in Section 4.2.16, the arsenical moiety in the drug may also participate in the recognition of the binding site as the state of oxidation is important for the uptake of melarsen oxide. When the $K_i$ value for melamine was calculated (Figure 3.43), the figure obtained ($7.0 \times 10^{-6}$M) was lower than the $K_t$ value ($14.5 \times 10^{-6}$M melarsen oxide); this is possibly due to the minor uncompetitive component found when studying the mechanism of inhibition of uptake of melarsen oxide by melamine.

4.2.19 The effect of different arsenicals on parent and resistant lines

The sensitivity to other arsenicals was tested in both parent and resistant lines. The structural analogues of melarsen oxide used were para-aminophenyl arsenoxide, phenyl arsenoxide and sodium arsenite.

The motility tests performed on both lines of the parasites (Table 3.11) showed that para-aminophenyl arsenoxide and phenyl arsenoxide are still active against the resistant line. Inorganic arsenite did not have any effect on parent and resistant lines. Furthermore when uptake studies were performed on the different structures mentioned above (Figure 3.40) it was found that phenyl arsenoxide was transported equally effectively by both lines; however para-aminophenyl arsenoxide is taken up to a lesser extent by resistant line organisms.

The sensitivity of the melarsen oxide resistant line to neutral aromatic arsenicals is of relative importance as it has been reported that a sodium melarsen resistant line developed by Rollo and Williamson (1951) was resistant to arsenicals of the neutral aromatic type (Table 1.4). Two alternatives are put forward to explain the differences observed. The first one related to the oxidation state of the arsenic moiety is untenable, as it has been reported that this moiety needs to be in the reduced form before any uptake can take place. Furthermore it was observed in this thesis that the pentavalent sodium melarsen is not taken up by the parasite (Section 3.6.10); both observations are not consistant with the proposition. The second alternative takes into account the method for the production of the resistant line used by Rollo and Williamson (1951) as the trypanosomes were exposed to the drug with the relapse method; with this routine the parasites are under the continuous influence of the drug after inoculation of the infected hosts until a new trypanosome relapse develops and the parasites are transferred to a new host. The possibility is that the continuous influence to the drug may have operated in such a way that the selective pressure allowed only the development of organisms with resistance to the entire active agent and hence the cross resistance with neutral aromatic arsenicals.

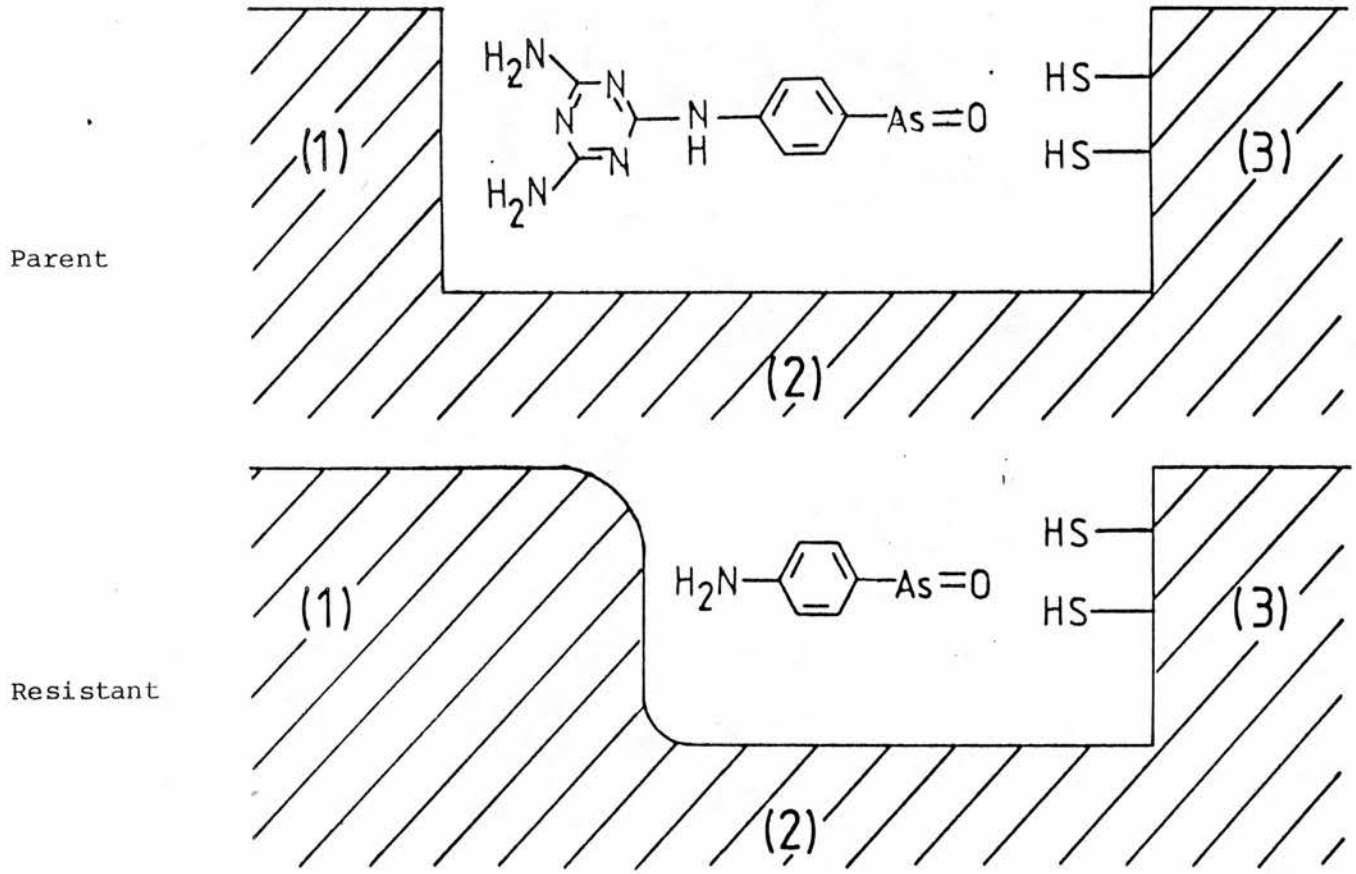
With the method used in this thesis, the short passage, the trypanosomes are taken from the exposed animal to drug untreated hosts before a considerable reduction in the parasitaemia has occurred, therefore the selective drug pressure may be lower than the drug pressure exerted using the relapse method. Thus the organisms did not develop resistance to the entire drug but to the melaminyl part of melarsen oxide. This alternative is only an inference as more experimental data is needed to substantiate it.

Regarding the transport of the different arsenicals studied it is observed that phenyl arsenoxide was taken with a high rate of uptake when compared with the other structures, although the high trypanocidal effect of the drug immobilised the trypanosomes very quickly even at very low extracellular drug concentration (Table 3.11). As for melarsen oxide a concentrative effect is observed for the drug; it is suggested that the drug is apparently taken up by a carrier mediated mechanism but due to the high trypanocidal effect associated with the drug, the saturation of the carrier cannot be observed under the conditions used. Further discussion on this carrier mechanism is presented below.

The second structure studied para-aminophenyl arsenoxide shows a clear saturation effect in both parent and resistant line; furthermore a differential uptake is apparently operating in the resistant line as a higher $K_m$ for the drug is observed (Section 3.40). The feature is interpreted as a carrier mechanism with less affinity for the drug. Damper and Patton (1976b) found that among the characteristics developed for the transport of pentamidine in a resistant line of T. brucei to this drug was a lower affinity for the drug. This characteristic modification of active transport mechanisms with loss of affinity for the drug is also observed in different cell lines derived from cancer tumours (Goldman, 1973).

The observation that the transport system of para-aminophenyl arsenoxide has been affected by the development of resistance, as shown by partial resistance to the drug, suggests that this structure is sharing the same binding site for melarsen oxide in the parasite. It is more difficult to decide whether or not phenyl arsenoxide is showing the same transport mechanism with the other drug studied. Similar results to those presented here were obtained by Hawking (1937) and Eagle and Magnuson (1944) when working with T. rhodesiense and T. equiperdum respectively; they reported that aromatic arsenic resistant lines of these parasites did not absorb organic arsenicals containing an amide group, but still absorbed and were inhibited by arsenicals having either unsubstituted phenyl arsenoxide or carrying the substituents Cl or $CH_3$. It appears that the unsubstituted phenyl arsenoxide may have a separate site of uptake; this proposition is untenable as it means that more than one normal site of uptake is being shared by different organic arsenicals (see Section 4.2.13). Instead it has been suggested (King and Strangeways, 1942) and still quoted by Adrient(1973) that compounds like phenyl arsenoxide, without any other hydrophilic group, enter the cells due to its lipid solubility. This latter observation is difficult to substantiate as it has been observed in this thesis that: 1) there is a possible involvement of the trivalent arsenic moiety in the binding of the drug, 2) the concentrative effect observed for this drug, an observation inferred from the results of Hawking (1937) on the transport of this drug, 3) the high rates of uptake at which the drug is taken from the extracellular compartment and 4) the structural similarities with the other drugs studied. Therefore it is concluded that the uptake of phenyl arsenoxide is carrier mediated, and furthermore that the transport mechanism is the same as for melarsen oxide and para-aminophenyl

Figure 4.2: Tentative model for the binding site of some arsenical drugs at the membrane level in T. brucei.




Three main sites of interaction are proposed for the tentative model; site one is considered to be specific for the melaminyl residue in melarsen oxide; site two which is apparently involved in the interaction with the aromatic residue of the arsenicals tested (melarsen oxide, para-aminophenyl arsenoxide, phenyl arsenoxide) and site three with possible involvement of -SH groups. On development of resistance to melarsen oxide, site one is apparently modified. This modification may also involve some change in site two of the model

arsenoxide with the difference that the development of resistance to melarsen oxide is not able to modify the transport of this structure. A similar conclusion to that presented here is reached when studying the uptake of different diamididdines in T. brucei (Damper and Patton, 1976b).

Inorganic arsenite was not taken up by any of the parasites (Figure 3.40) so it is assumed that the organic part of the molecule is needed for the uptake. Further studies on the mechanisms of inhibition of the transport systems of the drugs studied are needed to substantiate the inferences postulated with respect to the transport of arsenicals in T. brucei, as these studies may confirm whether or not the same active site is used for the different drugs. Some technical difficulties have to be overcome in order to complete these studies: 1) very rapid techniques for exposure of the cells to the drug and the respective transport inhibitors are needed, as both would be active trypanocidal agents; 2) specific assay systems must be developed to estimate the levels of the particular drug studied. The technique used in this work for the estimation of arsenicals could not differentiate between the inhibitor and the permeant and misleading results would be obtained.

4.2.20 A model for the binding site for the transport of some organic arsenicals

In spite of the lack of evidence on the modes of competition of the different drugs used, a hypothetical model for the site of transport of some organic arsenicals may be suggested to resume the results obtained with the transport studies.

As observed in Figure 4.2 the model has three main sites. Site one is suggested to participate in the binding of the melaminyl part of the drug and has been shown from the competition studies to

be specific for the binding of this structure. This site is mainly affected by the development of resistance to melarsen oxide (Figure 3.46(1)). Support for this proposition is obtained from the cross resistance studies of Williamson and Rollo (1959), which implied that the resistant character to melaminyl arsenicals was developed towards the amidine linkages (=N-C=N) in the triazine ring of the drug and that this modification was at the level of drug binding by the parasite.

Site two of the model intervenes in the binding of the aromatic residue of the drug; it is suggested that this site is affected only to a limited extent with the development of resistance. As shown in this work, the substituent in the para-position of the phenyl arsenoxide may serve as evidence to illustrate this point. It was also found that the melarsen oxide resistance line was partially resistant to para-aminophenyl arsenoxide (Figure 3.40). Again some preliminary evidence is found in the work of Williamson (1959a) when studying the effect of antagonists on the action of different arsenicals in vivo and in vitro. It was found that although melamine was a good antagonist of melaminyl arsenicals (see above) para-aminobenzoic acid also showed similar properties as melamine but to a lower extent; due to the structural similarities of this antagonist and the aromatic part of melarsen oxide it is suggested that the effect of the former is probably produced by competition for site two of the binding site. Mud (1945) commented on the importance of the para-aminophenyl residue that there were structural similarities of a series of compound with this particular structure i.e. sulphonamides, local anaesthetic esters of para-aminobenzoic acid and organometallic antiprotozoal agents. The argument was further discussed by Williamson (1959a) as he suggested the existence of a common and widely distributed cellular reaction site for such structures.

At this stage it is important to stress the need for a binding site shared not only by melaminyl arsenicals but also by aromatic arsenicals and which also explains the partial resistance mentioned above with respect to para-aminophenyl arsenoxide. A single binding site will explain as well the findings of Williamson and Rollo (1959) regarding the complete cross resistance between sodium melarsen and other aromatic arsenicals; and also the lack of cross resistance to melaminyl arsenicals showed by an atoxyl resistant line of T. rhodesiense. A modification of site two in the model will not necessarily impair the uptake of melaminyl arsenicals, which as shown in this work needs the melaminyl residue for an effective binding. Also this residue increases the affinity of the binding site for the drug when compared with the amino substituted phenyl arsenoxide. Using the same reasoning, the activity of melaminyl arsenicals against tryparsamide resistant trypanosomes reported by van Hoof (1947) can be explained.

On the other hand melaminyl resistant trypanosomes may or may not show cross resistance with aromatic arsenicals, as this will depend on the extent to which the trypanosomes are exposed to the drug during the development of the resistant character. This suggestion is made on the basis of the comparison reported in Section 4.2.19 on the development of melaminyl arsenical resistant lines using different methods.

Site three of the model seems to involve the presence of available -SH groups. This observation is inferred from the lack of uptake of the pentavalent melarsen oxide when compared with the trivalent analogue; this characteristic of the model will also explain the lack of uptake of pentavalent tryparsamide in T. rhodesiense (Hawking, 1937). Inorganic arsenite was not apparently taken up under the conditions

of rapid exposure to drug used in this work. However long term exposure to the drug showed that the drug is taken up and is lethal to the parasites (Hawking, 1937). It has been found that this arsenical reacts with simple dithiols such as dimercaprol but not to monothiols such as glutathione (Aldrigde and Cremer, 1955) hence it is proposed that site three may have at least two -SH groups for the binding of trivalent arsenicals.

It is clearly understood that the model proposed is relatively complex in its structure. However further investigation in relation to its validity may be useful from the rational chemotherapy point of view, as any information derived from it may help to increase the understanding of the mechanism of action of organic arsenicals at the level of binding to the parasite.

4.2.21 Summary

For the purposes of studying the biochemical rationale behind the development of resistance to arsenical drugs, a melarsen oxide fast strain of T. brucei was developed using the short passage method. The strategy followed in the study, was to compare the basic metabolism of the new strain with the parent line as a primary examination from which further alternative explanations for resistance could be proposed. Although it was found that the fast strain of T. brucei developed a minor modification in the basic metabolism of carbohydrate, this difference could not explain directly the levels of resistance observed with the fast organisms. Detoxication of arsenicals by antagonism with -SH groups was studied and no differences were found between parent line and the resistant strain.

One alternative explanation for the development of resistance is by modification of the enzyme targets to the drug in the resistant strain. Pyruvate kinase and phosphoglycerate kinase were studied in relation to

their kinetic parameters, specific activities and sensitivity to the arsenical drugs and no differences were found that can be suggested as causes of the development of resistance to the drug.

The transport of the arsenicals in the parent line and the resistant strain of T. brucei was also studied. It was found in the first place that the uptake of melarsen oxide by the parent line showed saturation kinetics with a concentrative effect, high temperature dependance, inhibition by iodoacetate, competitive inhibition by a structural analogue and finally that -SH groups may be involved at the drug binding site. As a result of this evidence it was concluded that the drug was transported by a carrier mediated active process. Furthermore, the kinetic results show a complex mechanism of transport in terms of the interaction with the membrane.

Regarding the development of resistance, it was observed that the fast strain lost the capacity for active transport of the drug and instead a simple diffusion system is responsible for the uptake of low levels of melarsen oxide. As no direct evidence of modification was found when testing the other alternatives, the differential drug uptake is concluded as being responsible for the development of resistance. It is inferred that the carrier mechanism is modified in the resistant strain and either absent, or can no longer bind the drug.

Further studies on the uptake of other organic arsenicals suggest the possibility of a single binding site at the membrane level for the drugs tested. A modification of part of the binding site is apparently responsible for the development of resistance to melarsen oxide; this modification does not necessarily alter the uptake of other drugs using the same carrier. Further studies are suggested on the structure of the transport mechanism, if possible with a more sensitive method of estimation for the drug.

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